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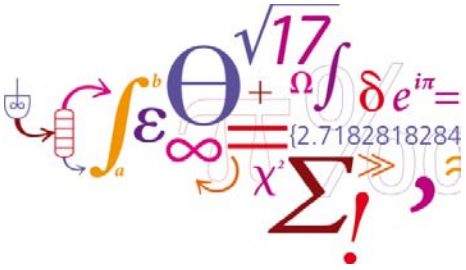
Process Technology for Immobilized Lipase-catalyzed Reactions

PhD Thesis

Yuan Xu

March 2012



DTU Chemical Engineering
Department of Chemical and Biochemical Engineering

Preface

This thesis is written in partial fulfillment of requirements for the degree of Doctor of Philosophy (PhD) at the Technical University of Denmark (DTU). The work has been carried out in the Center for Process Engineering and Technology (PROCESS) at the Department of Chemical and Biochemical Engineering from March 1st 2009 to 29th Feb 2012 under the supervision of Professor John M. Woodley and Dr. Mathias Nordblad.

This project is part of Sustainable Biodiesel project, collaboration between DTU Chemical and Biochemical Engineering, DTU Management Engineering, Novozymes A/S, Emmelev A/S and Aarhus University, funded by the Danish National Advanced Technology Foundation.

It is a great pleasure to have the opportunity to work on this sustainable biodiesel project with many nice and intelligent people from universities and companies. First of all, I would like to sincerely thank Professor John M. Woodley and Dr. Mathias Nordblad for their tremendous help and inspiring guidance on my Ph.D study. Their knowledge and experience have not only contributed to the outcomes of this PhD project, but also educated me to think more logically and creatively. I believe all I have learned from them will be highly useful to my future career.

I also wish to express my gratitude to Jesper Brask, Per M. Nielsen, Anders Rancke-Madsen and Pavle Andric from Novozymes for sharing the knowledge as well as supplying enzymes, the key role of the project. I really want to show my appreciation to Per, Jesper and Professor Xu Xuebing because it is they who introduced me to this wonderful biodiesel world since my master project.

Fruitful results and novel ideas have also been contributed by students who have joined the biodiesel project in the past three years. I specially wish to thank Rui, Hema, Anders, Lene, Lasse, Mette, Birgitte, Cesar for their outstanding contributions.

I want to thank a group of special and interesting people in PROCESS. Like dear friends, they make my every working day happy in the past three years. These colleagues compose the best memory I have had. Finally, I am grateful to the support from my family in China all the years.

Abstract

Biocatalysis has attracted significant attention recently, mainly due to its high selectivity and potential benefits for sustainability. Applications can be found in biorefineries, turning biomass into energy and chemicals, and also for products in the food and pharmaceutical industries. However, most applications remain in the production of high-value fine chemicals, primarily because of the expense of introducing new technology. In particular lipase-catalyzed synthesis has already achieved efficient operations for high-value products and more interesting now is to establish opportunities for low-value products. In order to guide the industrial implementation of immobilized-lipase catalyzed reactions, especially for high-volume low-value products, a methodological framework for dealing with the technical and scientific challenges and establishing an efficient process via targeted scale-down experimental work is described in this thesis. The methodology uses economic targets to test options characterized via a set of tools.

In order to validate the methodology, two processes based on immobilized lipase-catalysis have been studied: transesterification and esterification of vegetable oils for the production of biodiesel. The two processes are focused on the conversion of the two main components of vegetable oil materials, glyceride esters and free fatty acids respectively, into fatty acid alkyl esters. Although biodiesel is conventionally prepared via chemical-catalyzed transesterification of vegetable oils with methanol to produce fatty acid methyl esters (FAME), this work has been focused on the production of fatty acid ethyl esters (FAEE) with bioethanol due to the expected improved sustainability of this type of biodiesel.

A key reaction characteristic of the immobilized lipase-catalyzed transesterification is that it is multi-phasic system. The by-product glycerol can potentially impose inhibitory effects on immobilized lipases and likewise the un-dissolved ethanol can inhibit the lipase. The options for addressing these issues can be used as the basis for selecting the biocatalyst and the reactor (e.g. a hydrophobic carrier for the immobilized lipase and the capabilities to provide sufficient mixing as well as stepwise/continuous feeding of ethanol to the reactor).

An STR is efficient for batch operation while a PBR is efficient for a continuous production. An STR can more easily provide sufficient external mass transfer for a reaction, but will lead to more mechanical damage of the biocatalyst particles, than a PBR. A reactor combination

of CSTR with PBR can couple the advantages of both, delivering an efficient continuous process.

The second case study (esterification) shares some similar process characteristics to the first case (e.g. the multi-phasic nature). However, instead of glycerol, water shows a great impact on the extent of reaction. The removal of water should therefore be feasible during the operation of the reactor, either intermittently or preferably *in situ*. Highly anhydrous reaction conditions and the smaller substrates for this reaction place particular requirements on the lipase.

In order to validate the established processes at a larger scale, both lipase-catalyzed transesterification and esterification developed in the lab-scale STRs have been carried out in pilot-scale STRs. Results in both scale STRs correlate well with respect to the biocatalyst performance and mechanical stability.

Once the technical and scientific challenges of the process have been addressed, it is of course important to evaluate its economic and environmental feasibility. To that end, process evaluation has been performed for six processes composed of transesterification and product purification for making ‘in-spec’ biodiesel and the conventional chemical process is taken as a bench mark for comparison. The optimal process is a process composed of lipase-catalyzed transesterification with ‘in-spec’ biodiesel product as output with less feedstock input and waste production and much saved energy from the absence of product purification.

Dansk Resumé

Biokatalyse har tiltrukket sig betydelig opmærksomhed i den senere tid hovedsagelig på grund af meget høj selektivitet og potentielle bidrag til en mere bæredygtig produktion. Anvendelsesområder findes inden for bio-raffinaderier, hvor biomasse konverteres til energi eller kemikalier, samt for produkter i fødevare og farmaceutisk industri. De fleste anvendelser optræder dog indenfor produktion af høj-værdi kemikalier, hovedsagligt fordi det er omkostningstungt at introducere ny teknologi. Man har især opnået effektiv produktion af høj-værdi produkter med lipase-katalyseret syntese, og interessen er nu også samlet om at etablere muligheder for lav-værdi produkter. For at guide den industrielle implementereting af katalyserede reaktioner med immobiliserede lipaser, særligt for høj-volumen og lav-værdi produkter, præsenterer denne afhandling en systematisk metode for at imødegå de tekniske og videnskabelige udfordringer, og etablere en effektiv produktionsproces via målrettet eksperimentelt arbejde på nedskaleret udstyr. Metoden benytter økonomiske kriterier for at teste muligheder karakteriseret ved en række værktøjer.

For at kunne validere den systematiske metode, studeres to processer som er baseret på katalyse med immobiliserede lipaser, omestring og esterficering af planteolier til produktion af biodiesel. De to processer er fokuseret mod konverteringen af de to hovedkomponenter i planteolier, glyceridestere og frie fedtsyrer til fedtsyre-alkylester. Selvom biodiesel konventionelt dannes ud fra en kemisk katalyseret omestringsproces af planteolier med metanol til fedtsyre-methylester (FAME), så fokuserer dette arbejde på produktion af fedtsyre-ethylester (FAEE) ud fra bioethanol pga. den forbedrede bæredygtighed af denne type biodiesel.

Et meget vigtigt karakteristika er, at katalytisk omestring med immobiliserede lipaser er et multifasesystem. Biproduktet, glycerol, og uopløst ethanol kan potentielt inhibere af de immobiliserede lipaser. Denne viden kan benyttes som basis for valg af biokatalysator og reaktor, som for eks. et hydrofobt bæremateriale til den immobiliserede lipase, mulighed for tilstrækkelig omrøring og trinvis/kontinuer tilførsel af ethanol til reaktoren.

En omrørt tankreaktor er effektiv under batch drift, mens en pakket reaktor er effektiv under kontinuer drift. En omrørt tankreaktor kan levere effektiv ekstern masseoverførsel til reaktionen, men vil forårsage større mekanisk skade på biokatalysatorpartiklerne end ved

reaktion i en pakket reaktor. Med en kombination af en omrørt og en pakket reaktor kan man opnå fordelene ved begge, hvilket giver en effektiv kontinuer proces.

Det andet case study (esterificering) deler visse karakteristika med processen i den første case, fx tilstedeværelsen af flere faser. I stedet for glycerol er det vand, der giver en kraftig påvirkning af omsætningsgraden i processen. Det er derfor væsentligt, at vand kan fjernes fra processen enten trinvist eller endnu bedre *in situ*. De vandfri reaktionsbetingelser og det mindre substrat forbundet med denne proces, stiller nogle specielle krav til lipaserne.

For at kunne validere opførslen af de to processer i større skala, er forsøg i pilotskala udført for både omestringen og esterficeringen i en omrørt tankreaktor, som var udviklet på basis af en laboratorieskala omrørt tankreaktor. Resultaterne fra forsøg i begge skala korrelerer fint i forhold til ydeevne af biokatalysatoren og den mekaniske stabilitet.

Efter at have behandlet de tekniske og videnskabelige udfordringer, er det også væsentligt at evaluere de økonomiske og miljømæssige forhold. Til dette formål er procesevaluering udført for seks processer bestående af transesterficering og oprensning for produktion af kommerciel biodiesel. Den konventionelle kemiske proces er benyttet som sammenligningsgrundlag for disse. Den optimale process består af et lipasekatalyseret omestringstrin med kommerciel biodiesel som produkt, med et mindre substratinput og spildproduktion samt betydeligt mindre energiforbrug pga. den sparede oprensningsproces.

Abbreviations

ASTM	American society for testing and materials
BCR	Bubble column reactor
BSTR	Batch stirred tank reactor
CalB	*Candida antarctica* lipase B
CSTR	Continuous stirred tank reactor
DAG	Diacylglyceride
EBR	Expanded bed reactor
EtOH	Ethanol
FAEE	Fatty acid ethyl esters
FAME	Fatty acid methyl ester
FBR	Fluidized bed reactor
FFA	Free fatty acid
HPLC	High-performance liquid chromatography
ISPR	*In situ* product removal
MAG	Monoacylglyceride
MeOH	Methanol
N435	Novozym 435
NIR	Near-infrared
PBR	Packed bed reactor
PID	Process and instrumentation diagram
PMMA	Polymethylmethacrylate
SFS	Substrate feeding strategy
TAG	Triacylglyceride
TLL	*Thermomyces lanuginosus* lipase

Definitions

Enzyme activity	Units of product formed per unit time
Enzyme stability	The life time of enzyme (the time for a certain amount of enzyme to lose all activity)
Enzyme productivity	The mass of product formed per enzyme mass during the life time of enzyme
Space-time-yield	The mass of biodiesel product formed per volume of the reactor and time

Biocatalysts

Lipozyme TL IM	TLL immobilized on silica
Lipozyme RM IM	*Rhizomucor miehei* immobilized on macroporous anionic exchange resin
N435	CALB immobilized on a macroporous divinylbenzene-crosslinked polymethylmethacrylate
NS 88001 or NS 40077	TLL immobilized on a polymeric resin (an experimental catalyst)

Nomenclature

Re	Reynolds number	Dimensionless
U_t	Tip speed	m/s
P/V	Specific energy dissipation rate/power input per volume	W/L
N	Stirring speed	rpm
T_m	Mixing time	s
D	Impeller diameter	mm
H	Reactor height	mm
T	Reactor diameter	mm
C	Impeller position to bottom	mm

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CHAPTER 1

Introduction

1.1 Industrial biotechnology

'Industrial sustainability' was first introduced to public in the 1990s and has been interpreted as the continuous innovation, improvement and use of clean technologies to reduce pollution levels and maximizing the output from resources, preferably renewable resources (Sang and Ryu, 2005). Industrial biotechnology is believed as a key to this achievement because it uses living cells and enzymes to produce goods and services from renewable resources with a smaller influence on the environment and lower production costs of energy, water and capital investment (Villadsen, 2007). Additionally, this technology is advantageous in the synthesis of products which are chemically intractable (Hatti-Kaul et al., 2007). Industrial biotechnology has its wide applications in biorefineries, turning biomass to energy, chemicals, as well as food and pharmaceutical industries. It can even be found in upcoming nanotechnology field (Sang and Ryu, 2005).

As part of the industrial biotechnology, biocatalysis is defined as using either isolated or immobilized enzymes as catalysts for the synthesis of chemical products. Most studies concerning biocatalysis have been using single enzyme while multienzyme catalysis is also getting attractive for the production of many compounds at an industrial level (Santacoloma et al., 2011). Multienzyme processes can be arranged with two or more enzymes in a cascade, a parallel, or a network configuration to drive the synthesis towards a primary product (Cornish-Bowden, 2004).

Industrial implementation of biocatalysis is more active in the synthesis of high-valued fine chemicals due to its characteristics, such as high reaction selectivity, improved product purity, simplified reaction process (reduced reaction steps) but limited catalytic abilities of biocatalysts, cost-intensive production of biocatalyst, lack of support from bioprocess technology and so on (Tufvesson et al., 2010, Hatti-Kaul et al., 2007). Examples of industrial applications of biocatalysis are shown in Table 1.1. To expand its implementation, solutions are being sought in the evolution or exploration of robust biocatalysts, which can perform efficiently under tough conditions as many industrial processes present. This is where the research and development of protein engineering and enzyme engineering has contributed to the rapid growth of biocatalysis (Schmid et al., 2001).

Table 1.1 Application examples of biocatalysis in industry (Jensen and Rugh, 1987; Shewale and Sivaraman, 1989; Leuchtenberger et al., 1984; Xu, 2003; Tufvesson et al., 2010)

Product	Enzyme	Annual production (ton)
High fructose corn syrup	glucose isomerase	> 1 000 000
lactose-free milk	lactase	> 100 000
acrylamide	nitrilase	> 10 000
cocoa butter	lipase	> 10 000
nicotinamide	nitrilase	> 1 000
6-aminopenillanic acid	penicillin amidase	> 1 000
L-methionine, L-valine	aminoacylase	> 100
ampicillin	penicillin amidase	> 100

There are also non-technical barriers limiting the implementation of biocatalysis, which are mostly related to some misconceptions about enzymes, for example, enzymes are very unstable of low productivity and enzymes can easily cause allergy. These worries are something that learned from the earlier days of enzyme technology. Nowadays, the enzyme technology has been greatly developed. Some immobilized enzymes can be stable for years, e.g. aspartase and isomaltulose synthase (Rozzell, 1999). The enzyme can be safely used by changing the formulation. Nevertheless, a better understanding of the advantages and limitations of enzymes is important to the effective use of enzymes.

1.2 Lipase catalyzed reaction

Lipases (EC 3.1.1.3) belonging to hydrolase class, are versatile in catalyzing a variety of reactions. Hydrolysis and ester synthesis are two basic lipase-catalyzed reactions and similarly lipases can catalyze transesterification of esters with alcohols. In addition, acidolysis and interesterification can also be catalyzed by lipases via consecutive basic reactions (hydrolysis of ester followed by esterification), as shown in Figure 1.1 (Jegannathan et al., 2008). The catalytic mechanism of lipases is often described by the ping-pong bi-bi model, releasing each product between each addition of substrate (Paiva et al., 2000).

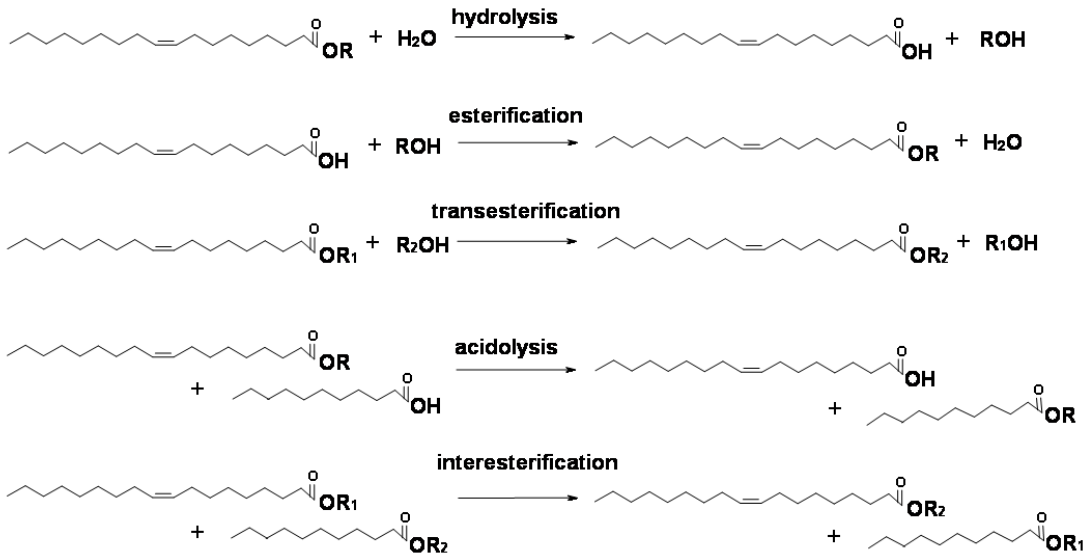

Figure 1.1 Classic reactions catalyzed by lipase (Hayes, 2004)

The most accepted explanation about the catalytic ability of most lipases is the 'lid' theory, which is described as a mobile element with a flexible structure covering the catalytic site of lipases when they are not activated. The lid composed of a α-helix region in the active form can open and expose the binding site to substrates and the activation of lipase is restricted to a water-lipid interface. Even though the reaction medium is hydrophobic, the small water pools near the active site can serve as the local interface for a configuration change of the enzyme (Pleiss et al., 1998; Balcáo et al, 1996). This theory applies to most lipases. However, *Candida antarctica* lipase B is one of those exceptions of the 'lid' theory because it lacks the lid domain and is not activated at water-lipid interfacial surface probably due to the large hydrophobic surface surrounding the entrance channel of the active site (Uppenberg, et al., 1994; Martinelle et al., 1995).

Lipases are capable of producing various products with high purities and consequently high added value due to the different substrate specificities including fatty acid specificity, positional specificity and stereo-specificity (Song et al., 2008).

Some lipases show strong selectivity for short-chain esters, like *Candida antarctica lipase* B (CalB) whereas some for medium-chain or long-chain fatty acid esters, and some for branched esters. CalB also catalyzes the acylglycerols likely in the order of MAG > DAG > TAG (Watanabe et al., 2001; Xu et al., 2011). The esterification of FFA with MeOH catalyzed by CalB was more than 10 times faster than methanolysis of TAG (Watanabe et al., 2007a).

Some lipases can selectively hydrolyze the ester bond at position 2 of the triglycerides, e.g. *Candida antarctica* A while some, e.g. *Rhizopus oryzae* lipase and *Thermomyces lanuginosus* lipase (TLL), have the specificity for 1 and 3 positions (Douchet et al., 2003; Song et al., 2008; Du et al., 2005). That can probably explain that TLL catalyzes TAG faster than MAG and DAG (Xu et al., 2011).

Lipases from *Penicillium cyclopium and Chromobacterium viscosum* show a distinct preference for 1-*O*-octadecyl-*sn*-glycerol over its enantiomer indicating a stereoselectivity for the *sn*-3 position (Meusel et al., 1992).

These different substrate specificities vary significantly dependent on the sources of the lipase, most likely because of the differences in their structures, especially the structures of active sites. For example, CalB has an elliptical, steep funnel-like binding site of limited space while TLL has a crevice-like binding site of larger space, which can probably explain their different specificities for acylglycerols (Pleiss et al., 1998; Türkan and Kalay, 2006).

Like many other enzymatic reactions, lipase can catalyze reactions under mild conditions of temperature and pH, representing the natural metabolisms. Therefore, those reactions involving temperature sensitive reactants and products become possible with lipase catalysis (Balcáo et al., 1996).

1.3 Lipase formulation

Several forms of lipase have been used for biocatalysis. Lipase can be kept inside the host cell to perform the biotransformation (whole-cell catalysis) and it can be used as an isolated enzyme (biocatalysis in free or immobilized form). The biocatalytic process can be affected by the lipase form in many ways, i.e. catalyst stability, selectivity and mass transfer as well as the production cost because the preparation costs of different formulations are significantly varied (Tufvesson et al., 2011).

Whole-cell

The preparation of whole-cells is simple and cheap. It can be in free form or more often in the immobilized form. However, whole cells can involve unwanted side reactions and could suffer from mass-transfer limitations. Furthermore, the whole-cell catalysts generally have limited compatibility with organic solvents and high concentrations of substrate or product

(Tufvesson et al., 2010). Whole-cell catalysis is regarded as biotransformation (using resting cells) and so it is not the focus of this work.

Liquid formulation of lipase

Lipases are soluble in aqueous solutions and such a formulation of lipase makes the phase condition less complex (only liquid), which theoretically could catalyze a faster reaction than the whole cell and immobilized lipase by avoiding the complex mass transfer through multiple phases (Nielsen et al., 2008).

The preparation of liquid formulation of lipases is less complicated and of lower cost compared to immobilized lipases but still more expensive than whole-cell preparation because the recovery and purification of lipase are costly (Tufvesson et al., 2011).

Immobilized lipases

The operational stability of lipase can be improved by the immobilization of an enzyme, which can allow the reuse of the catalysts and simplify the downstream processing of the product (Christensen et al. 2003).

The immobilization techniques have been rapidly developed recently and many of them have been applied on lipases, including cross-link, encapsulation adsorption and covalent linkage to carriers (Christensen et al., 2003). For the purpose of choosing the proper technique for lipase immobilization, one important fact which should be kept in mind is that lipases usually work with fats under water-limited condition or with the presence of organic solvent. Therefore, the techniques should be able to retain water for the catalytic efficiency since lipases in general need the interface to work.

Immobilized lipases are expensive because the carrier as well as the immobilization process, adds significantly to the cost of immobilized enzymes (Nielsen et al., 2008). Due to the high cost of the immobilized lipases, they are required to have remarkable productivities particularly when they are applied in producing low-value bulk chemicals or biofuels, normally in a magnitude of tons product per kg immobilized lipases.

1.4 Lipase applications

The abovementioned catalytic advantages entitle lipases wide potential applications in many industries. However, they have been commercially used in only a few industries. The biggest market for lipases is the detergent industry and the other major applications are

nutropharmaceutical related food industry, the dairy products, chiral pharmaceutical compound and fine chemical industries (Balcáo et al., 1996). Novozym 435 (N435) is probably most well-known immobilized lipase and has been widely studied in academic research. It is *Candida antartica* B lipase immobilized on macroporous divinylbenzene-crosslinked polymethylmethacrylate (PMMA). However, due to its expensive carrier and complicated immobilization procedure, its application is typically restricted to such high-value products as cosmetic esters and omega 3-boosted fish oils. It also displays a high stereoselectivity, extensively applied in synthesis of chiral products for pharmaceutical use. Lipozyme TL IM which is *Thermomyces lanuginosus* lipase (TLL) immobilized on silica, a hydrophilic and cheaper carrier, is commercially used for the production of trans-free margarines and shortenings (Holm and Cowan, 2008; Lee et al, 2004).

The reason that existing lipase applications are mostly limited to valuable products is likely related to the high cost of lipase production. It is still in their infancy of the industrial applications of lipase in producing low valued products, such as biodiesel. To commercialize more industrial applications of lipases, the efforts can focus on the progress of protein engineering to increase enzyme activity and stability. The cost of lipase can be reduced by improving the downstream technology for enzyme purification. Additionally, the development of process technology for biocatalysis can also be a key to this matter by optimizing the reaction conditions and maximizing the reaction efficiency.

1.5 Bioprocess technology

Biocatalytic processes are different from conventional chemical process in many aspects, such as enzyme kinetics, protein stability and bioactive features derived from the biological hosts (Schmid et al., 2001). Therefore, the traditional process technology based on chemical processes is not competent to serve the biocatalytic processes. Thus the development of process technology based on biocatalytic process should be stimulated in order to match the progress of biocatalysts. With the development of bioprocess technology the biocatalytic processes can be optimized to be economically viable so that biocatalysis can also be applied in the synthesis of high-volume low-valued bulk chemicals and biofuels. The sustainable aspects of biocatalysis can be more appreciated by such applications due to the increased scale.

Biocatalysis is typically characterized as heterogeneous reactions (Schmid et al., 2001) and biocatalysts are more vulnerable to inhibitions than chemical catalysts, which necessitate specific tasks for bioprocess technology, such as the bioreactor design and operation, substrate supply and product removal, reuse of biocatalyst, process modeling and simulations, scale-up and process control. The major aspects of bioprocess technology are described in the following sections.

1.5.1 Reactor options

Multiphasic nature is a key characteristic of the immobilized lipase-catalyzed biocatalysis, which determines that the reactor choice has to fall within the category of solid-liquid contacting reactors. They are introduced in three categories with classic reactor examples.

1.5.1.1 Well-mixed reactors

STR is a typical well-mixed reactor meaning ideally no concentration gradient spatially inside the reactor. STRs are the most often-used reactors for biocatalysis at different scales because of the ease of construction, operation and maintenance (Balcáo et al., 1996). They can be operated in both batch and continuous modes (Buchholz et al., 2005).

The mechanical damage to the immobilized enzymes is one of the major disadvantages of STR, which affects the reusability of the biocatalyst and raises the risk of contaminating the products (Halim et al., 2009).

STRs are adaptable to almost all processing needs and objectives with flexible configuration designs and various choices of impeller, baffling and their positions inside the tank. The optimal performance requires a careful matching of reactor configuration and tasks.

Turbine impellers are often applied for low to medium viscosity fluids and they are categorized to two major types according to the flow pattern they create: axial flow impellers and radial flow impellers. Characteristics and examples are given in Table 1.2. Axial flow impellers can circulate the flow in an axial direction, which are efficient at blending and suspending solids, while radial flow impellers are normally used for liquid-liquid blending because of the higher shear and turbulence levels provided by this type of impeller (Paul et al., 2003). As the immobilized lipases are sensitive to high shear, axial flow impellers with

down-pumping are more appropriate, although many reported studies use Rushton turbine, a classic radial flow impeller.

Table 1.2 Turbine impellers for STR (Paul et al., 2003)

Impeller type	Example	Characteristic	Purpose
Axial flow	Propeller, hydrofoil	Low power, high pumping, a single circulation loop	Solid suspension, blending
Radial flow	Rushton turbine, Flat-blade impeller	High shear, low pumping, compartmentalization of circulation	gas-liquid and liquid-liquid dispersion
Mixed flow	Pitched blade turbine	Balance of pumping and shear capability	General-purpose

When the ratio of liquid height and tank diameter is high (>1.3), multiple impellers are needed to provide the sufficient mixing. As a rule of thumb, the dished-bottom tank is preferable for solid suspension to the flat-bottom tank or the conical-bottom tank (Paul et al., 2003). For the flow in turbulent or transitional regime, baffles are highly recommended to break the vortex. Wall baffles are sufficient to lift the solid catalysts and can transform tangential flows to vertical flows creating top-to-bottom mixing (Paul et al. 2003).

Table 1.3 Application examples of STR in immobilized lipase-catalyzed reactions

Immobilized lipase	Reactants	Co-solvent	Product	Impeller	Reactor scale	Operation mode	Reference
Lipozyme RM IM	Palm oil, oleyl alcohol	n-hexane	Wax esters	Rushton turbine	2 L, 75L	Batch	Keng et al., 2008
N435	Decanoic acid, glucose	t-butanol	Glucosylde canoate (sugar esters)	60° axial flat blade impeller	1L	Batch	Han et al., 2011
N435	Adipic acid, oleyl alcohol	no	Dioleyl adipate	Rushton turbine	0.5L 2.5 L	Batch Continuous	Chaibakh sh et al., 2010
Lipozyme TL IM	Palm stearin, coconut oil	no	Margarine fat		1 L, 300 L	Batch	Zhang et al., 2001

The application examples including the biodiesel application of this work are given in Table 1.3. Most of examples are for high-viscosity reaction mixtures and radial impellers are widely used mainly for liquid blending. Those applications are still operated in batch mode of lab or pilot scales.

1.5.1.2 Plug-flow reactors

As a classic plug flow reactor, PBR presents a concentration gradient along its length. The conversion efficiency of a PBR in terms of its length, behaves in a manner similar to that of a batch STR in terms of its reaction time. PBR is basically composed of a column with frits in both ends to retain catalyst particles and auxiliary equipments, such as a water bath for maintaining required reaction temperature, pumps for transferring reactants.

PBR provides a larger reacting surface area per unit volume than an STR and is often applied in continuous industrial processes with a high volumetric productivity (Balcáo et al., 1996). PBR is preferred for reactions involving reaction reversibility and product inhibition because the product concentration is minimized due to the plug-flow characteristic. Additionally, a PBR gives a lower shear stress to the catalyst particles than an STR and simplifies the separation of biocatalyst from product (Halim et al., 2009).

On the other hand, the major disadvantage of PBR is the high pressure drop which can be associated with small packing-material size, high flow velocity or obstruction of the catalyst bed by accumulation of insoluble components from the reaction mixture. Therefore, fairly rigid immobilized-enzyme particles are required in PBRs because high pressure drop may distort compressible or physically weak particles and the compressed or deformed particles can result in a reduced catalytic surface area and channeling.

The other drawback of PBR is the mass transfer limitations, which is because the flow pattern inside PBR can be restricted due to the limited flow velocity relative to the pressure drop allowance. PBR also has the difficulty of adding substrate, which however can be solved by sequentially running a number of PBRs with intermittent substrate addition (Nielsen et al., 2008).

1.5.1.3 Mixed-type reactors

There are some heterogeneous reactors of mixed behaviors of a well-mixed reactor and a plug-flow reactor. One such example is a series of well-mixed reactor e.g. STRs to simulate

the plug flow system, as illustrated in Figure 1.2. In such an assembled reactor system, a gradual transformation undergoes and the gradient is composed of several constant conversion levels.

Furthermore, this design allows the intermittent substrate feeding and product separation between tanks, which is highly useful for reactions having substrate or production inhibition by keeping low concentrations of them in each reactor.

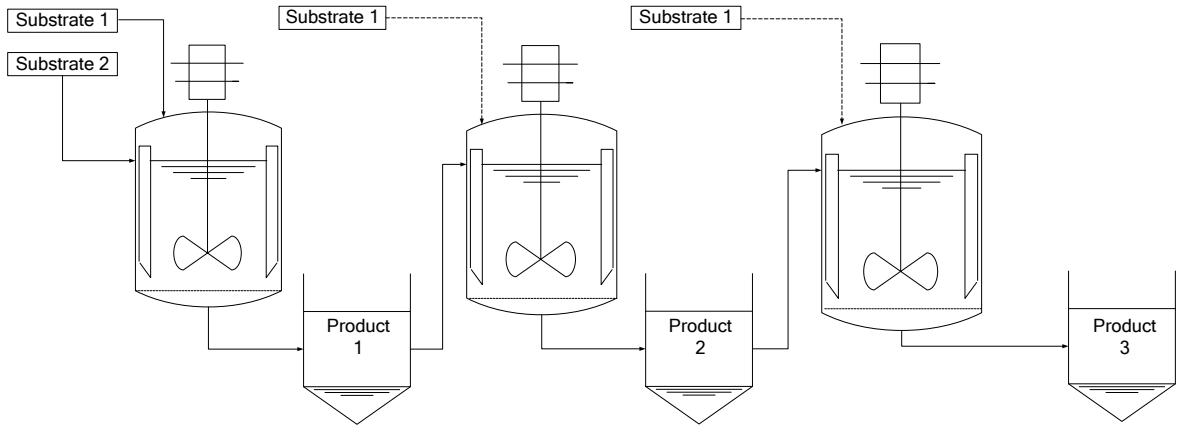

Figure 1.2 Mixed-type reactor composed of a series of identical STRs

1.5.2 Reactor operations

According to the continuity of the process, the operations of reactors can be classified as batch, fed batch and continuous mode. All reactors are subject to batch operations and are also possible to be run in a continuous manner with some modifications to configurations. The operation mode of the reactor should be selected specifically according to the process and the task.

1.5.2.1 Batch operation

It is a simple but labor-intensive way of operating reactions, adding all reactants in stoichiometric proportions to a reactor to start the reaction and removing the product after the reaction. Such batch operations need to be repeated again and again to complete the production task. It is commonly used in small scale plants or labs. Batch operation needs less equipment investment and offers flexibility but reduces the volumetric productivity due to the downtime involved between batches for emptying and cleaning reactor and adding fresh

substrates. It is difficult to ensure the consistent product quality through a large number of batches (Balcáo et al., 1996).

1.5.2.2 Fed batch operation

Fed batch operation derives from a strategy originally for fermentation of feeding growth limiting nutrients to control cell culture. It is adopted more widely as a substrate feeding strategy (SFS), and featured by continuously adding one or more substrates to the reaction system when enzyme can be inhibited by a high concentration of substrate or undissolved substrate. This operation permits more stable and safer operation than a batch operation.

1.5.2.3 Continuous operation

Continuous operation has almost the opposite characteristics to batch operation. It has less operational cost and a higher efficiency for large scale production. More reproducible products can also be expected from the constant reaction conditions of continuous operations.

The advantages of immobilized enzymes can be most remarkably appreciated in continuous operations because it eliminates preparing catalyst in the feeding stream due to its retainable heterogeneous form and it is easier to maintain the constant catalyst concentration in the reactor than homogeneous catalysts.

1.5.3 Reactor scale up

A scale-up effort is to obtain the equivalent process performance or result at a larger scale as the same process in a bench scale. A successful scale-up is based on the good understandings of the challenges and opportunities provided from bench scale experiments and a sound use of methodology, innovative ideas and preferably abilities of assuming business risks (Donati and Paludetto, 1997). Today about 150 biocatalytic processes are operated in large scales (Tufvesson et al., 2010) compared to a massive number of commercialized chemical processes. The difficulties of scaling up bioprocesses mainly come from the complicated system, e.g. the non-ideal or unknown fluid flow behavior, the unpredictable effects of environmental changes on biocatalyst.

As the most important part of the whole process, scale-up of reactors is the core step in the industrial realization of a process. The multiphasic nature of the immobilized lipase-catalyzed

reaction system requires the consistent fluid dynamics in different scales to achieve the similar mass transfer and reaction rates.

1.5.3.1 Scale up of STR

The empirical approaches for STR scaling up include geometric similarity, constant specific energy dissipation rate (P/V), equal tip speed (U_t) and constant flow pattern determined by Reynolds number (Re). The similar geometry is normally kept in the scaled reactor and one of the other above mentioned parameters also remains constant according to the specific reaction requirement, which is often the most important factor to the reaction.

No matter using any approach, it is however always difficult to maintain the similar hydrodynamic conditions at different scales. Therefore, it is necessary to be aware of the deviation and evaluate its influence on the biocatalyst performance before choosing the approach. A common change of STR scale-up is the increased mixing time (circulation time). Thus, the effect of time-dependent variables on the performance needs to be evaluated (Tufvesson et al., 2010).

Table 1.3 shows these variances of mixing parameters correlated to common-used approaches for STR scale-up by a factor of 5 in impeller diameter, reactor diameter and height. Owing to the geometric similarity, the mixing time (T_m) acts inversely as the change of the stirring speed (N). As the stirring speed is always reduced at the scaled reactor, increased mixing time is an inherent problem of scaling up STR. For those fast reactions where mixing time is critical, it is necessary to keep the stirring speed constant but it however results in very high P/V at a larger scale, 25 times higher in this case (Table 1.3). A huge power input is not acceptable to most processes, which makes it more sense to keep the same P/V for the scaled STR. The consequences of this strategy are the more vigorous shear stress to the biocatalyst and the changed flow pattern because of the increased tip speed (U_t) and the increased Re value. If the particles are sensitive to the shear damage, the constant U_t can be used but the mixing takes longer.

After comparing these approaches, the constant P/V or the constant tip speed should be the most appropriate strategies for the immobilized lipase-catalyzed process because the resulting changes of mixing quality are relatively smaller and acceptable.

Table 1.4 Important changes in mixing parameters and fluid dynamics on scale-up of STR from lab-scale to pilot-scale by different criteria (D, H and T change by 5-fold)

N	P/V	U_t	Re	Tm
1	25	5	25	1
0.34	1	1.7	8.6	2.9
0.2	0.2	1	5	5
0.04	0.008	0.2	1	25

1.5.3.2 Scale up of PBR

When scaling up a PBR for a larger application, pressure drop is always an important concern because it determines the feasibility, operational safety and the power input. According to Darcy's law, the pressure drop is proportional to the length, the viscosity of the fluid and the flow velocity but varies inversely with packing material size (Bird et al., 2002). Therefore, the PBR is often scaled up width-wise in order to avoid the length associated pressure drop issue. On the other hand, this type of scaling way will be restricted to the extent that wide columns may vary from plug flow, with significant dispersion. Smaller packing material can give an increase in the higher reaction surface but it will greatly increase the pressure drop. Therefore, the optimal particle size should be a balance of both these issues.

As a rule the superficial velocity is always kept constant for scale up since it is such an important parameter which determines the mixing efficiency inside the PBR, particularly when viscous components can cause mass transfer limitations.

1.5.4 Substrate feeding strategy (SFS) and *in-situ* product removal (ISPR)

SFS and ISPR strategies can be beneficial to reactions catalyzed by immobilized lipases which suffer from the substrate inhibition or product inhibition. SFS is able to maintain the optimal level of substrate at the interface required by the lipase while ISPR can reduce the concentration of inhibitory products. They are also capable to shift the reaction equilibrium by affecting the concentration of substrate or product to improve the conversions.

Applying SFS can not only control the inhibitory substrate beneath the critical concentration, also in some cases improve the selectivity (e.g. stereoselectivity) of biocatalyst by reducing the substrate concentration (Watanabe et al., 2000; Houng and Liau, 2003).

Common SFS can be seen with reactor operations, e.g. fed batch, or via auxiliary methods, such as utilizing a second phase (organic solvent, ionic liquid or resin) to form a demand-based delivery system (Kim et al., 2007).

The physico-chemical properties, such as volatility, solubility, hydrophobicity and molecular size, are most often utilized for ISPR (Lye and Woodley, 1999). The relevant ISPR strategies include evaporation of volatile product, liquid–liquid extraction, adsorbing resins and membrane extraction (Shin and Kim, 1997; Truppo et al., 2010; Yun et al., 2004; Shin et al., 2001).

1.6 Objectives and challenges of the thesis

Although lipase-catalyzed synthesis has achieved efficient operations for high-value products, few examples can be found in large scale productions of low-value products. It is therefore necessary and interesting to establish a suitable methodology for guiding the process development of immobilized lipase-catalysis for making high-volume low-value products. The methodological framework aims to deal with the technical and scientific challenges and establish an efficient process via experimental work directed by an industrial target. The methodology is expected to be verified by two examples: immobilized lipase-catalyzed transesterification and esterification for biodiesel production.

The challenges of implementing these two processes are presented as follows:

The industrial implementation of immobilized lipase-catalyzed reactions require remarkable productivities of the biocatalysts to achieve the economic feasibility of the processes. A remarkable productivity is normally determined by the operational stability and the mechanical stability of the immobilized enzyme. Unfortunately, the lipases can be inhibited by both substrates and products and the carriers of immobilized lipases are sensitive to mechanical stress as reported (Watanabe et al., 2002; Xu et al., 2011; Keng et al., 2008). Therefore, one of the challenges in this project is to keep the immobilized lipases both operationally and physically stable by utilizing the bioprocess technology.

The other challenge for the chosen case studies is making products meet the biodiesel specifications, which has seldom been reported so far but is highly desirable to the process although it is not favored by the thermodynamics. It raises the question that how to draw the reaction to the desirable side by bioprocess technology.

The scale up of the process is also a big concern of this project. It is important to find out that how the hydrodynamic conditions change at scaled reactors and how they influence the biocatalyst performance.

1.7 Structure of the thesis

The main body of the thesis comprises nine chapters and a short introduction of each chapter is given as follows:

Chapter 1 provides comprehensive information of the background of implementing lipase-catalyzed synthesis. It introduces the lipase-catalyzed reactions and their applications on the stage of industrial biotechnology and the topic extends to the potentials of bioprocess technology for expanding the industrial implementation of lipase-catalyzed synthesis to low-value products. An overview of bioprocess technology is presented with selective details in this chapter.

Chapter 2 proposes a systematic methodological framework for industrial implementation of immobilized lipase-catalyzed reactions. The framework is composed of a number of steps and tools are suggested for each step to guide the experimental work.

Chapter 3 presents the background information on biodiesel. The important issues associated with feedstock and alcohol are addressed and possible solutions are also discussed in this chapter. The methods of producing biodiesel (chemical and enzymatic) are reviewed and compared. The purpose of this chapter is to give an overview of the biodiesel production and help understand the case studies in the next chapters.

Chapter 4 demonstrates the application of the proposed methodology in an example of immobilized lipase-catalyzed transesterification for making biodiesel. Processes have been established in two reactors in the lab and the STR process has been validated in a pilot plant.

Chapter 5 describes a second example applying this methodology in the immobilized lipase-catalyzed esterification for polishing the biodiesel product. A PBR process has been established in the lab and ‘in-spec’ product has been obtained. A lab-scale STR process has been evaluated and tested in the pilot plant.

Chapter 6 evaluates the established processes together with other process options in light of the mass and energy balances, which can reflect the economic and environmental feasibilities. The conventional chemical biodiesel process has been taken as a benchmark.

Chapter 7 discusses the other opportunities of applying this methodology for process development in enzymatic biodiesel industry. Practical issues of applying the methodology are addressed. Some suggestions are also given in this chapter to improve the process efficiency.

Chapter 8 provides the most important findings of the case studies and the proposed methodology.

Chapter 9 proposes work for the future on both the studied cases and the methodology.

The results and details of process development of the two studied cases are provided in four papers, which are included in Appendix D of the thesis.

The reaction characteristics and enzyme kinetics have been studied and an important *in-situ* dyeing method has been developed for indicating by-product glycerol in Paper 1. Paper 2 has evaluated the process in a lab-scale stirred tank reactor (150 mL) and validated this process in a pilot-plant scale (20 L). To investigate the potential of industrial production, continuous operation has been studied in both packed bed reactor and stirred tank reactor as presented in Papers 3 and 4.

CHAPTER 2

Methodology

2.1 Introduction

A systematic methodology for developing immobilized lipase-catalyzed process has not been found in any published work. The lack of methodology raises the difficulties of establishing and industrially implementing such processes. As a major objective of this PhD thesis, a methodological framework is proposed and to be validated with concrete examples of immobilized lipase-catalyzed reactions. The methodology aims to provide guidelines for selecting biocatalysts and reactors for a given immobilized lipase-catalyzed reaction and identifying the key constraints of designing and operating a bioprocess via experimental work.

2.2 Methodology description

The proposed methodological framework is outlined in Figure 2.1 including the pathway and tools suggested for each step.

2.2.1 Step 1 Establish reaction scheme

As a starting point, a reaction scheme needs to be established as a basis for developing the process, which requires identifying the substrates and products. For a given kind of reaction substrates can be chosen from different sources and products are subsequently determined.

Project objectives can guide this selection of substrate. For instance, the economic targets and environmental considerations limit the choices of substrates because the substrates (raw materials) can contribute greatly to the total production cost. The availability of raw materials is also an important factor to be considered when choosing substrates. An easy access to the raw materials is definitely an advantage to the success of the whole process.

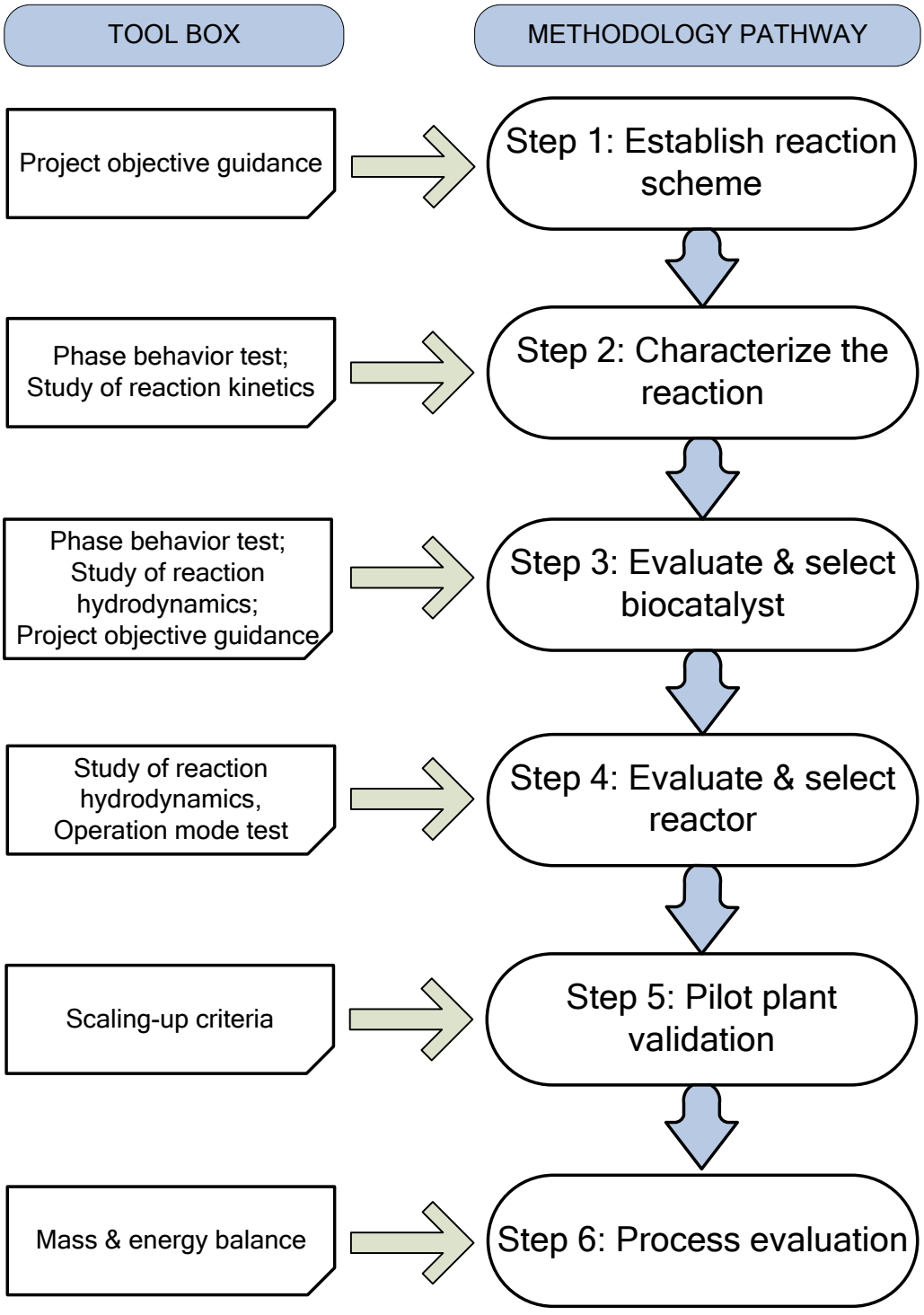

Figure 2.1 Outline of the methodology for implementing immobilized lipase-catalyzed reactions

2.2.2 Step 2 Characterize the reaction

Identification of the key characteristics of the reaction system can provide criteria for biocatalyst and reactor selections and define the process constraints. Such information can be obtained from literature searching or experimental investigation. The following tools are suggested for characterizing the reaction via experiments. Figure 2.2 illustrates how these tools contribute to the reaction characteristics and explanations are given in the following paragraphs.

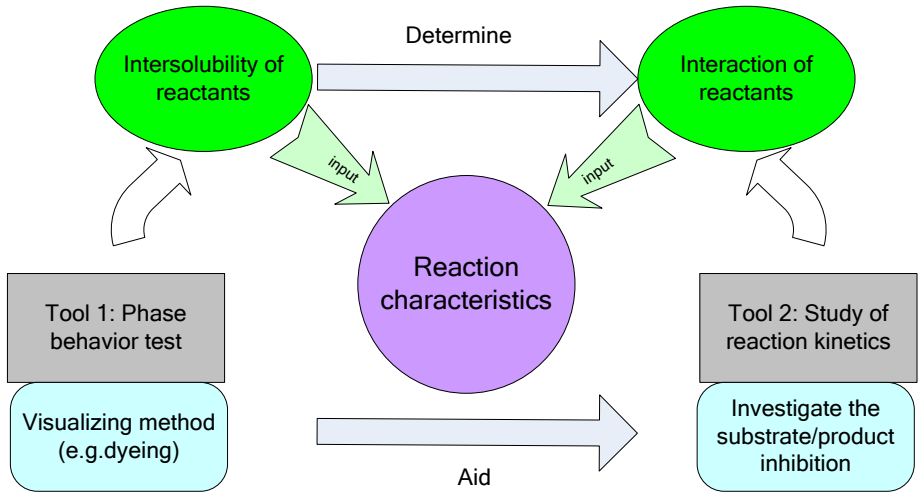

Figure 2.2 Illustration of characterizing the reaction

Tool 1: Investigation of phase behavior during the reaction (phase behavior test)

The first step is to check the intersolubilities of substrates and products, which can be studied in an actual reaction system by preparing a mixture of substrates and biocatalyst and providing basic reaction conditions (temperature, agitation, etc.) or in a simulated reaction system by mixing major reactants. The former approach checks the ongoing process, closer to reality and presenting the effect of intermediate reactants on the phase behavior of the reaction system while the latter approach is easy to manipulate the concentrations of reactants mimicking different extents of the reaction.

This is particularly relevant to immobilized lipase-catalyzed reactions because they are always multi-phasic reaction systems, the reactants of which can be hydrophobic lipids, hydrophilic alcohols, glycerol and water as well as the insoluble phase of the immobilized lipases themselves. Thus, the investigation of the intersolubilities of reactants can help to understand the interactions of the reactants and the requirements for reaction conditions.

Some methods should be developed to facilitate the study of phase behavior, preferably able to visualize the phase formulation for a fast evaluation, for example, dyeing a specific phase or reactant.

Tool 2: Study of reaction kinetics

The reaction characteristics can also be obtained by looking into the interactions of biocatalysts with substrates and products, e.g. substrate inhibition and product inhibition. The experiments for studying the effects of substrates can be conducted, for example, by keeping the lipase loading and the concentration of one of the substrates constant and altering the concentration of the potentially inhibitory substrate, which can be learned from scientific literature or results from phase behavior test. The effect of product can be studied by adding different amounts of the suspected product which can possibly cause problems to the immobilized lipase. These effects can be presented by analyzing the initial rates of the reaction and/or the yield of target product after a fixed reaction time.

2.2.3 Step 3: Evaluate and select biocatalyst

As an important role in the given reaction system, immobilized lipases need to be carefully selected to fulfill the task of the project. The previous steps in reality narrow the choices. For example, the choice of substrates can suggest a range of lipases as some of them show particular substrate preferences. The identified kinetic constraints work as filters to eliminate unfavorable lipase candidates. Additionally, some other tools can be applied at this step. Figure 2.3 shows how these tools work in this step and details are given as follows.

Tool 1: Phase behavior test

The interactions of immobilized lipases, especially the carriers and other reactants obtained from the previous step can define some necessary properties that the carriers should possess, e.g. less affinity for some inhibitory reactant. The same methods for phase behavior test can be used here again to select the optimal carriers of the immobilized lipases with the desirable hydrophobicities or the acceptable affinities for those problematic reactants.

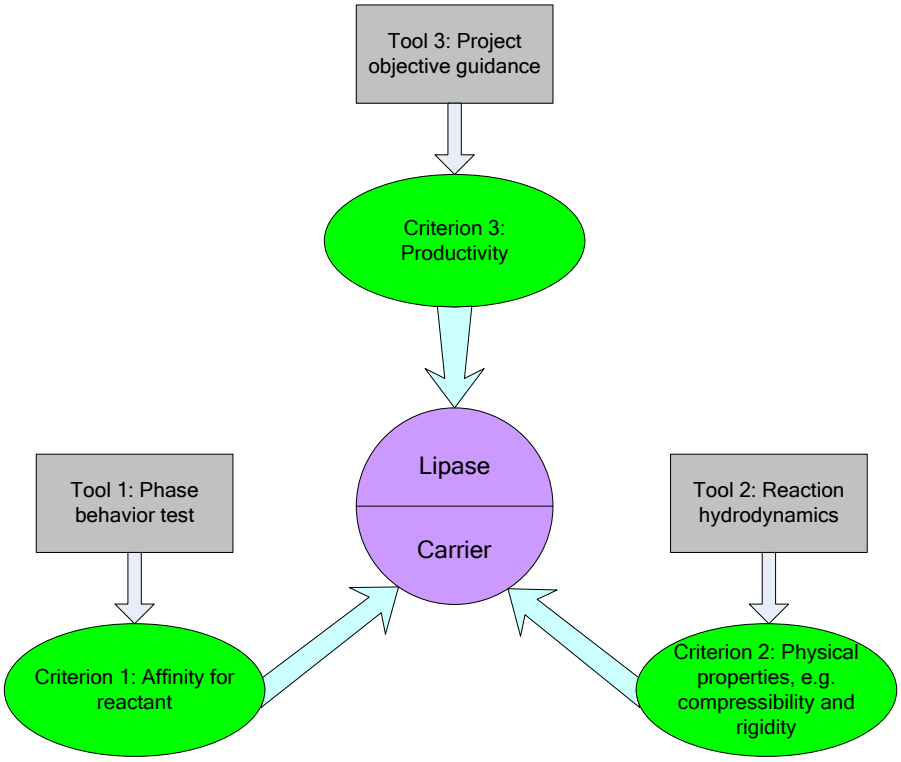

Figure 2.3 Tools for evaluating and selecting biocatalysts

Tool 2: Study of reaction hydrodynamics

The immobilized lipases require a certain fluid-solid hydrodynamic condition to catalyze the reaction and normally such a hydrodynamic condition is created by external force, very likely mechanical agitation. The carriers of immobilized lipases should be compatible with such hydrodynamic requirements and shear stress directly imposed by the external force.

Some experiments are necessary to perform to test the physical properties of carrier candidates. They can be designed to focus on the compressibility and rigidity of the catalyst carriers. If there is no easy access to the methods for such studies, these tests can be performed together with the reactor selection in the next step since the hydrodynamic condition is highly relevant to the choice of reactor.

Tool 3: Project objective guidance

The biocatalyst candidates can also be evaluated according to the requirement for lipase productivity assigned by the economic objective. The following equation can be used for calculating the productivity target (Tufvesson et al., 2011):

$$\text{Productivity target (ton product /kg biocatalyst)} = \frac{\text{biocatalyst cost (USD/kg biocatalyst)}}{\text{allowable cost contribution(USD/ton product)}}$$

The allowable cost contribution can be referred to the catalyst cost in the conventional production route, likely non-enzymatic route of the same product, or calculated via an economic assessment of the profit target, product price and market, cost of raw material, etc.

After defining the productivity target, experiments (e.g. operational stability test under standard conditions) can be carried out in a small scale to rapidly evaluate the productivity and compare it to the target to eliminate the unqualified lipase candidates.

2.2.4 Step 4: Evaluate and select reactor

For the immobilized lipase-catalyzed reactions, the reactor plays an important role as it accommodates the immobilized lipase and provides the hydrodynamic environment for the reaction to proceed. The previous assessments (e.g. identification of key reaction characteristics) put basic requirements for the reactor, which can be heterogeneous phase contact and mechanical agitation. Furthermore, the optimal choice of reactor should also allow to be scaled up preferably applicable to empirical scaling-up principles. A list of reactor candidates can be generated based on these considerations and they need to be individually evaluated.

Tools are suggested to aid the evaluations, as shown in Figure 2.4. Worthy to note, it is always relevant and more efficient to evaluate reactors within industrial limits. For example, the experiments for evaluating a stirred tank reactor is suggested to be designed within a power input per volume 1.0 W/L.

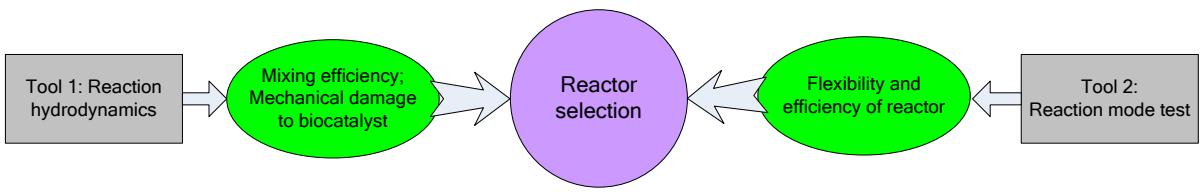

Figure 2.4 Illustration of evaluating and selecting reactor

Tool 1: Study of reaction hydrodynamics

The hydrodynamics of the reactor candidates are highly correlated to the performance of the immobilized lipase. It is because the hydrodynamic condition in the reactor can determine the mass transfer rates of substrates to the lipase through multiple phases as well as relieving substrate or product inhibition for the biocatalyst.

Experiments can be designed to check the effect of flow pattern on the biocatalyst performance in each reactor candidate. It can be done by for instance, varying the stirring speed in STR or changing the superficial flow velocity in PBR.

The hydrodynamic conditions created in the reactor can affect not only the catalytic lifetime also the physical life time of the biocatalyst and both of them affect the overall productivity of the immobilized lipase. Therefore, the mechanical stability of the biocatalyst should also be part of the study. Evaluation can be a comparison of the particle size distributions before and after experiencing the agitation in the reactor.

Tool 2: Operation mode test

Industrial implementation requires high space-time-yield of the immobilized lipase in the reactor. Continuous operation can avoid down time of batch operation and allow consistent product. It is therefore preferable if the reactor can be competent in continuous operation. The operation mode test can be designed to check the flexibility by comparing the efficiencies of both batch and continuous operations of the reactor.

2.2.5 Step 5: Pilot plant validation

The previous steps are normally carried out in small-scale studies, which are fast, cheap and of high throughput. Eventually the selected process needs to be validated in a larger scale, e.g. a pilot scale.

First of all, scaling up of the reactor is a major task of scaling up the whole process. It can refer to empirical scale-up principles for the selected type of reactor. Normally a key parameter should be kept constant for scaling up, which can be a key hydrodynamic requirement for the reaction or an operational constraint.

Second, in order to validate the process obtained from the small scale in a pilot scale, similar experiments should be conducted in the pilot scale under the same conditions. The reaction

kinetics in the pilot scale should be evaluated compared to those obtained in the lab scale, because it is a way to find out the difference of hydrodynamic conditions between scales.

Third, if deviations between scales are found, modifications should be carried out for the pilot-scale process.

2.2.6 Step 6: Process evaluation

A number of process layouts can hopefully be formed until reaching this step. They are advised to be evaluated in an industrial scenario (project objective) to check the economic and environmental feasibilities as the results can be directly compared to the expectations or budgets. Process evaluation can also be done by comparing the proposed processes to the conventional process as a bench mark to identify the improvements or bottlenecks of the proposed process options.

The relevant tools for this step can be calculations of mass and energy balance for possible process options. They are fairly useful to the economic profile of the process. The data used in calculations can be sourced from scientific literature and/or experimental results.

Some necessary assumptions are very likely needed to facilitate the evaluations and they will certainly introduce uncertainties into the evaluation, which should be discussed or adjusted afterwards.

Tool 1: Mass balance

To obtain the mass balance metrics, the process inputs (e.g. reactants, biocatalysts and reagents) and outputs (e.g. products, by-products and waste) should be laid out as Figure 2.5 shows. The calculation of these inputs and outputs can be based on a quantity of the product according to the industrial target. The resulting metrics can present the efficiency of converting substrates to the desired product in a given process.

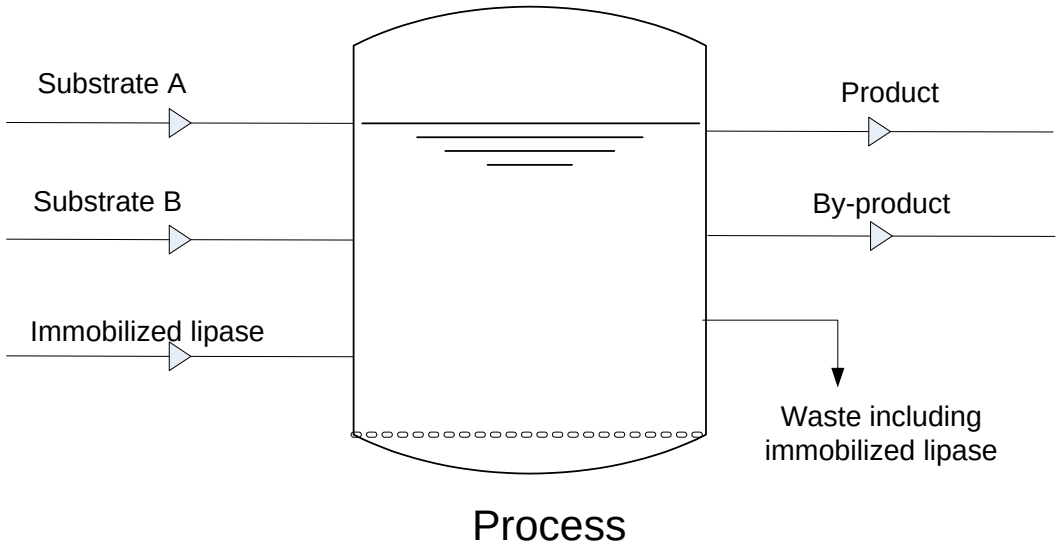

Figure 2.5 Illustration of material flow within an immobilized lipase-catalyzed process

Tool 2: Energy balance

Energy metrics are calculated based on energy inputs and energy outputs, which is dependent on the process design, more specifically the unit operations involved in the process. The operational conditions (temperature, pressure) are based on scientific literature and experiments. The heat capacity and enthalpy of vaporization of components at certain conditions can be looked up in databases, e.g. ICAS software (Gani et al., 1997).

When the energy output is low or not possible to be recycled, the calculation can be simplified by focusing on energy inputs, which would be certainly overestimated to different extent depending on the specific flowsheet. But they can be adjusted with more delicate calculations with considerations of the recycle of energy. Nevertheless, such simplified energy calculations can provide a quick comparison of energy consumption among different processes. The inputs of energy for an immobilized lipase-catalyzed process can come from two major parts, the reaction energy (e.g. agitation in the reactor, heating, pumping) and purification energy (e.g. energy-intensive distillation), as Figure 2.6 shows.

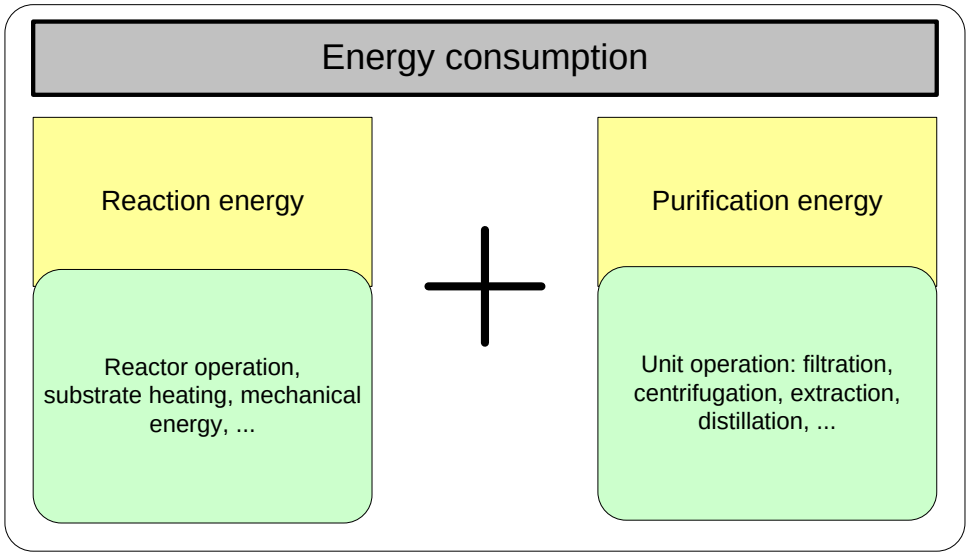

Figure 2.6 Components of energy consumption

CHAPTER 3

Introduction to case studies--biodiesel

3.1 Background of biodiesel

The depletion of fossil fuels and the associated pollution problems make it increasingly necessary to develop renewable energy alternatives that have smaller environmental impact than the traditional ones. Biodiesel shows great promise as a fuel and with respect to sustainability (Nielsen et al., 2008). Biodiesel is defined by the American Society for Testing and Materials (ASTM) as monoalkyl esters of long chain fatty acids derived from renewable feedstocks like vegetable oils and animal fats (Marchetti et al., 2008). It is still controversial if the definition should include other products, such as fatty acid ethyl esters (FAEE) (Lois, 2007).

For biodiesel producers it is very important for their products to meet the biodiesel standards. The commonly-used biodiesel specifications in USA and Europe (ASTMD 6751 and EN 14214), both show very low tolerance for impurities in biodiesel (Knothe, 2006). Table 3.1 shows the EN 14214 specifications for major component and purities in biodiesel.

Table 3.1 Specifications for major components of biodiesel from EN 14214 (wt%)

Parameter	FAEE	MAG	DAG	TAG	FFA
EN14214	min96.5	max0.8	max0.2	max0.2	max0.25

Biodiesel produced from vegetable oils or their blends have similar physical and chemical properties to conventional diesel fuel. It is the only alternative fuel to be used in existing diesel engines without modification. In fact, biodiesel has the following advantages over conventional diesel or petroleum. It is renewable, biodegradable, oxygenated and less toxic, and produces less smoke and particulates and lower CO_2 and less SO_x emission. It also has higher cetane number, low aromatic content and high heat content. Furthermore, biodiesel is less volatile and safer to transport or handle due to its high flash point (150 ^{o}C) (Robles-Medina et al., 2009, Akoh et al., 2007; Frondel and Peters, 2007). In addition, biodiesel can enhance engine yield and life because of its better lubricant properties (Vasudevan and Briggs, 2008).

3.2 Raw materials for making biodiesel

3.2.1 Feedstocks

Plant-derived oils are the main source for producing biodiesel, such as soybean, sunflower, cottonseed, rapeseed, palm oil, jatropha oil (Kumari et al., 2007). The fatty acid compositions of these vegetable oils are shown in Table 3.2. Among all the available vegetable oils, high oleic acid (C18:1) chain containing oils are favorable due to the increased stability on storage and improved fuel properties (Akoh et al., 2007). In this sense rapeseed oil is outstanding. The availability of vegetable oils for biodiesel production varies regionally dependent on the climate, soil conditions and farming traditions. Generally rapeseed oil, having the highest oil yield per acre land, is the dominating source for biodiesel production in Europe and so is soybean oil in the United States and South America. Owing to the low efficiency of catalysts (alkaline or lipases) working with crude oil, refined vegetable oils are the most suitable and widely used feedstocks (Van Gerpen et al., 2004; Behzadi and Farid, 2007). However, these high quality agricultural feedstocks are also used for food purposes. Consequently and unexpectedly, large areas of natural land, such as Amazon rainforest in Brazil which plays an important role in adjusting atmosphere, controlling soil erosion and pollution, has been explored to grow soybean or sugar cane, which raises the doubt about the net impact of biofuel on environment (Frondel and Peters, 2007).

Nevertheless, these controversial discussions about biodiesel bring attentions to the search of more sustainable approaches, for instance, the discovery of alternative nonfood-use feedstocks, like algae oil, waste cooking oil and other low-grade feedstocks.

At present, the cost of feedstock is the main hurdle of commercialization of biodiesel because it takes up 70-95% of the total cost of biodiesel production (Lai et al., 2005; Zhang et al., 2003). Waste cooking oil, a representative of low cost oil is very competitive from economic point of view as well as the ethical considerations about ‘food vs. fuel’ because it offers the opportunity of making use of surplus or waste biomass. Although the levels of FFA and water in waste oils (typically 2000 ppm water and 10-15% FFA) are manageable by enzyme catalytic process (Al-Zuhair, 2007; Nielsen et al., 2008), however, the source availability appears to be more critical to the potential of utilizing waste oil for industrial biodiesel application. The establishment of channels for collecting the wide spread sources needs the assistance and policy support from local governments.

Table 3.2 The fatty acid composition of possible oils for biodiesel (Akoh et al., 2007; Meng et al., 2009)

Oils	Fatty acid bound to glycerol backbone (%)							
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	other
Cottonseed	28.3		0.9	13.3	57.5			
Palm oil	42.6	0.3	4.4	40.5	10.1	0.2		1.1
Sunflower	7.1		4.7	25.5	62.4		0.3	
Soybean	11.4		4.4	20.8	53.8	9.3	0.3	
Rapeseed	35		0.9	64.4	22.3	8.2		
Jatropha	16.4	1.0	6.2	37.0	39.2		0.2	

On the other hand, genetic engineering can also help lower the cost of feedstocks in the near future as new crops will be developed with high oil content for nonfood use but this technology has not been well accepted world widely (Akoh et al., 2007).

Solutions to the hurdle of feedstock are seeking not only with the existing oil stocks also with the discovery of potential feedstocks. Algae oil is such a promising potential source for biodiesel production meeting the global demand for renewable fuels. It has high oil productivity with low cost because of its extremely fast growth and more efficient oil production than crop plants (Chisti, 2007). Additionally, the high content of polyunsaturated fatty acids may present stability problem due to the susceptibility to the oxidation during storage, but they also entitle algal biodiesel better cold weather properties than many other oils and fats because of the lower melting point (Demirbas, 2009). Today the technologies of algae cultivation, extracting and converting algae oil to biodiesel are not developing in accordance with each other. There is still a long way to go for the implementation of algae oil in biodiesel industry.

In summary, no practical solution has been found to solve the high cost of feedstock so far. Thus, it is more realistic to focus on developing the process technology to improve the biodiesel yield out of feedstock and utilizing low quality feedstocks, which is the achievement that this PhD work wants to obtain for the biodiesel case study.

3.2.2 Alcohols

Alcohols used in the production of generally defined biodiesel are primary and secondary monohydric aliphatic alcohols having 1-8 carbon atoms (Ma and Hanna, 1999). Methanol is most widely used to produce FAME in conventional alkaline biodiesel production because it is reactive, cheap and easy to recover (Akoh et al., 2007; Van Gerpen, 2004). Methanol derived FAME is universally recognized and accepted as biodiesel. However, methanol is so far still made from natural gas and it is toxic, polar with a low solubility in oil.

Ethanol is gaining attention as an alternative to methanol, since it is more environmentally friendly and renewable than methanol because it can be obtained from agricultural products by fermentation and it is less toxic (Al-Zuhair, 2007). Although ethanol derived FAEE has not been well accepted as biodiesel, the fuel properties are similar to FAME and some of them are even better than FAME. For instance, the extra carbon brought by the ethanol molecule slightly increases the heat content, the cetane number and reduces the cloud and pour points (Bozbas, 2008; Bouaid et al., 2007). The mass yield of FAEE can also be increased by approx. 5% of the biodiesel weight (Nielsen et al., 2008). Although the first generation bioethanol is currently most available, produced from the sugar and starch, the use of ethanol in biodiesel production can be more sustainable with the success of the second generation bioethanol (cellulosic ethanol).

3.3 Methods for production of biodiesel

Biodiesel can be produced by transesterification catalyzed by chemical catalyst (alkaline, acid) or enzyme, or non-catalytic transesterification under supercritical conditions (Al-Zuhair, 2007). Discussions in this thesis are only dedicated to the catalytic reaction routes, especially the enzymatic route. Depending on the feedstock quality and the choice of catalyst, the catalytic reaction routes differ in some processing modules, besides the transesterification.

3.3.1 Conventional chemical biodiesel production

Before leading to the application of lipases in the biodiesel-related oil processing industry, it is necessary to have a look at the conventional biodiesel production method, which nowadays still dominates the biodiesel industry. The method generally includes major process modules discussed in the following sections.

3.3.1.1 Oil degumming

Degumming is the first refining step of vegetable oils, removing phospholipids, mucilaginous gums and metal contaminants that are bound to phosphatidic acid (Dijkstra, 2010; Jiang, et al., 2011). Van Gerpen and Dvorak recommended the phosphorous content to be less than 50 ppm to avoid noticeable yield loss in the conventional chemical biodiesel production and refined vegetable oils are qualified in this sense since the industrial refining standards require the phosphorous content lower than 10 ppm (Yang et al., 2006; Van Gerpen and Dvorak, 2002). Additionally, oil degumming is also a production process for lecithin, which serves as emulsifier in the food and pharmaceutical industries (Ceci, et al., 2008).

3.3.1.2 Alkaline-catalyzed transesterification

The primary commercial process for biodiesel production today is alkaline-catalyzed transesterification of refined vegetable oils with methanol (McNeff et al., 2008). The product from this process is fatty acid methyl ester (FAME) which is universally recognized biodiesel. The alkali catalysts e.g. sodium hydroxide and potassium hydroxide are soluble with reactants and have to be neutralized after the reaction (Al-Zuhair, 2007). The chemical route has the advantage of high conversion rates within short reaction time, cheap catalyst and modest operation conditions (Leung et al., 2010). However, alkali-catalyzed process can have side reactions like saponification (a reaction between alkaline and free fatty acids), which can greatly affect the yield and quality of biodiesel product. Therefore, it strictly requires anhydrous conditions specially a problem with wet ethanol and feedstocks of low FFA content specially a problem with non-refined oils.

3.3.1.3 Pretreatment on FFA content of high acidic feedstocks

FFA content in feedstocks varies with sources and processing procedures. Refined rapeseed and soybean oils usually contain less than 2 wt% whereas around 10 wt% in animal fats and the waste cooking oil can contain up to 15 wt% (Nielsen et al., 2008). Therefore, the FFA contents in these non-refined oils and fats have to be reduced to below 2.5 wt% for the conventional biodiesel process (Leung et al., 2010). The FFA level can be lowered by distillation of FFA or converting FFA in the oil to biodiesel in a pretreatment step, which is normally an acid catalysis before the alkali transesterification (Zhang et al., 2003). The former solution will cause yield loss and both solutions need to deal with the whole amount of raw material (oil and the contained FFA). Therefore, an integrated solution is proposed

that FFA is distillated from high acidic feedstock and can be converted to biodiesel before it rejoins the major oil stream (Brask et al., 2011).

3.3.1.4 Product purification

After transesterification in the traditional process, the biodiesel products are rarely within the biodiesel specifications due to the short reaction time and thermodynamic limitations, which means that a product purification process is required. The conventional purification process is complicated and energy intensive, including neutralization of alkali catalyst, water washing biodiesel followed by FAME distillation and glycerol distillation, as illustrated in Figure 3.1. The resulting salts from neutralization of alkaline reduce the quality and value of by-product glycerol (Meher et al., 2006; Van Gerpen et al., 2004).

3.3.2 Enzymatic biodiesel production

The major difference of the enzymatic biodiesel production and the chemical process is the enzymatic transesterification. They are compared in Table 3.3.

Aside from that, they have similar process modules when using refined vegetable oil, as illustrated in Figure 3.1, including oil degumming, transesterification and product purification. However, when using high acid feedstocks, e.g. waste oil, the pretreatment is not necessary since FFA can be converted by lipases to biodiesel. Furthermore, the immobilized lipase-catalyzed process requires fewer unit operations in the product purification than the chemical process.

In short, the enzymatic process is more adaptable to feedstocks of different qualities, highly selective with fewer side products and more energy efficient. It produces less waste water and high-purity by-product glycerol. It also has the advantage of environmental friendliness.

These advantages make the lipase catalyzed biodiesel process promising with great potential to substitute the conventional biodiesel process after overcoming cost limitations.

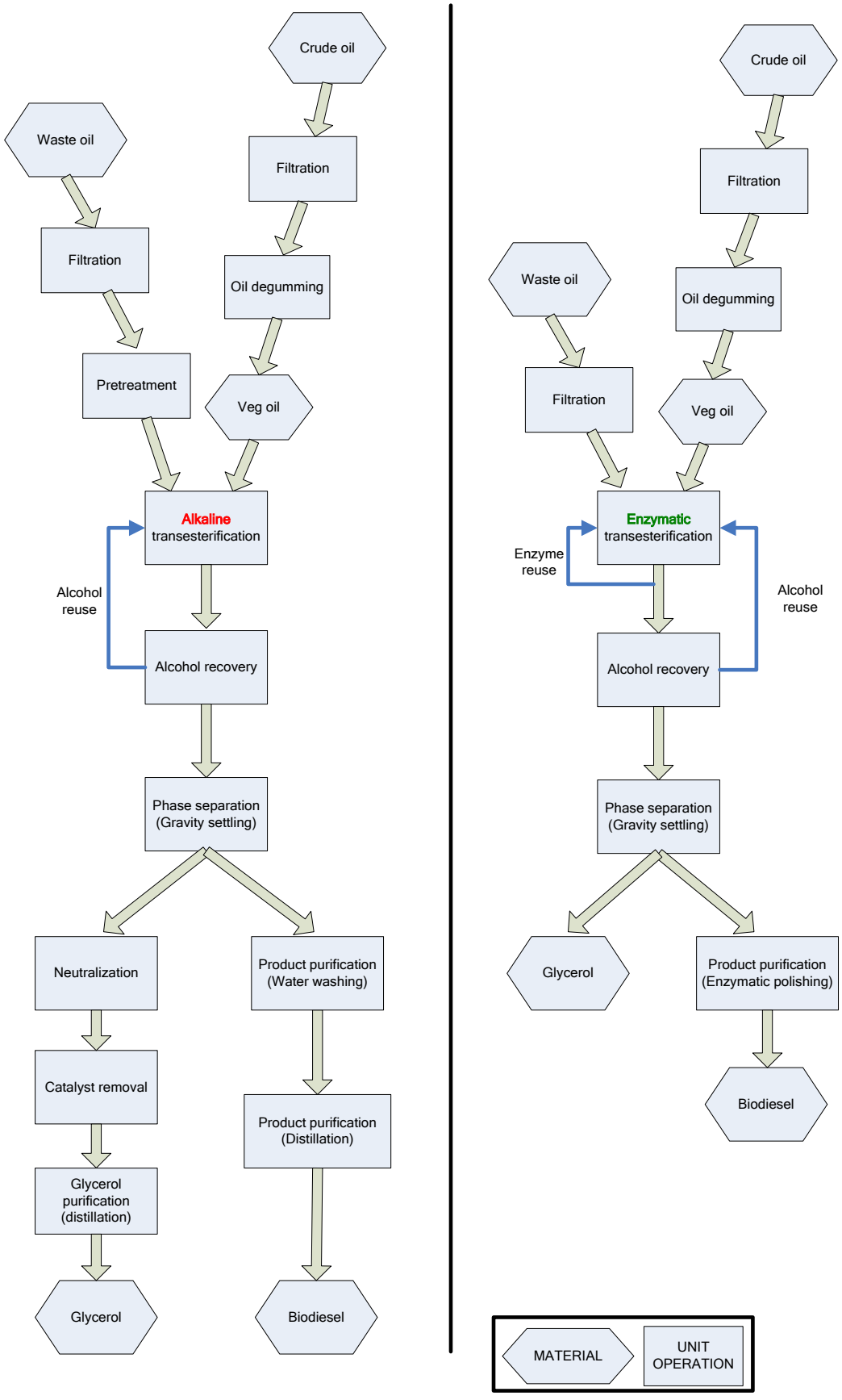

Figure 3.1 Chemical and enzymatic process for biodiesel production

Table 3.3 Comparison of alkaline- and enzymatic transesterification methods for the production of biodiesel (Van Gerpen et al., 2004; Watanabe et al., 2007b; Nielsen et al., 2008; Parawira, 2009)

Factor	Alkali catalysis	Enzymatic catalysis
Feedstock requirement	Low FFA and water	Flexible
Temperature	Medium and high	Low
Alcohol choice	Methanol	Methanol/ethanol
Batch reaction time	Short (1 h)	Long (6-24 h)
Product yield	Normal	Higher
Glycerol purity	Low	High
Catalyst cost	Low	High
Catalyst reuse	No	Yes
Commercialization	Yes	No

CHAPTER 4

Case study: immobilized lipase-catalyzed transesterification for making biodiesel

4.1 Establishing reaction schemes for enzymatic biodiesel production

Feedstock and alcohol are the major substrates for the transesterification and they can be chosen in a wide variety as mentioned in Chapter 3. According to the methodology, an industrial scenario can be used to define these elements such that they can be used to establish the relevant reaction scheme for given objectives. Subsequently, the enzymatic biodiesel production of a medium scale (annual 5 million gals, approx. 16000 tons) is proposed to take place in Brazil, where the biofuel industry has been stimulated recently by the government (Pousa et al., 2007).

As a consequence, the feedstock and alcohol can be focused on soybean oil and bioethanol, as they both have abundant production in Brazil. However, rapeseed oil has been used in actual experiments of this work due to a better accessibility to rapeseed oil in Europe than soybean oil and based on an assumption that rapeseed oil has negligible difference from soybean in the enzymatic process.

Besides the better renewability of bioethanol than methanol, bioethanol is also advantageous to the enzymatic process because the ethanol is less toxic and milder to the lipase (Kumari et al., 2007). Furthermore, the choice of bioethanol can also be made between 96% ethanol and absolute ethanol. The former is cheaper and as a azeotropic ethanol it can reduce the distillation burden when ethanol needs to be recovered in an industrial production. Therefore, 96% ethanol is preferred for a larger portion of the total ethanol consumption.

The resulting reaction schemes for this case are given in Figure 4.1. The FAEE-biodiesel is prepared via the stepwise transesterification of triglycerides and ethanol.

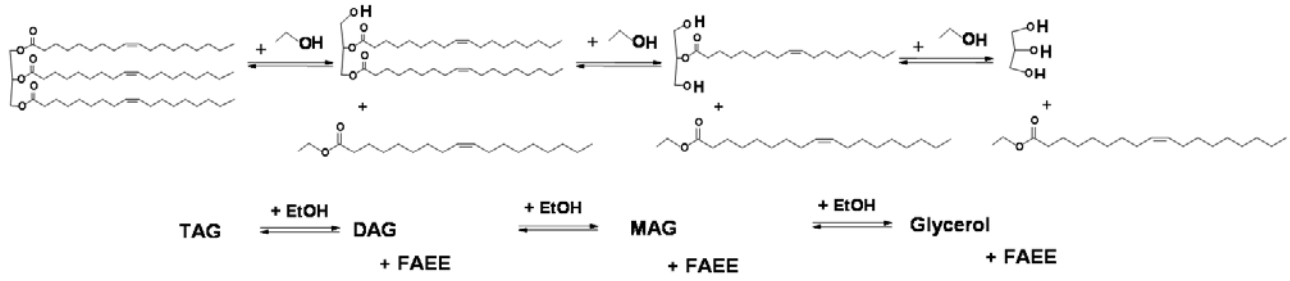

Figure 4.1 Reaction schemes of enzymatic transesterification using ethanol

4.2 Key reaction characteristics

The reaction characteristics of this case have been studied and presented in this section. They are fundamental and important as a platform for process development.

4.2.1 Multiphasic nature of the reaction system

According to the intersolubility test of reactants, the biggest characteristic of the immobilized lipase-catalyzed transesterification is the multiphasic nature, which is shown in Figure 4.2.

The reaction medium is mainly a lipid phase while the immobilized lipase introduces an insoluble phase all through the reaction progress. Although ethanol has a better solubility than methanol (Kumari et al., 2007), only 0.5 molar equivalent (eq) amount of ethanol to the total fatty acids can be dissolved in the oil at 35 ^{o}C. Therefore, the ethanol can add a polar phase on the top when it exceeds the solubility limit but this layer can disappear as the reaction goes on because the solubility of ethanol can be increased when intermediate reactants (e.g. DAG and MAG) are formed. As a by-product of transesterification, glycerol accounts for 10 wt% of the final product (Van Gerpen et al., 2004). It is immiscible with oil and biodiesel and has a higher density than any other component in the liquid phase of the reaction system.

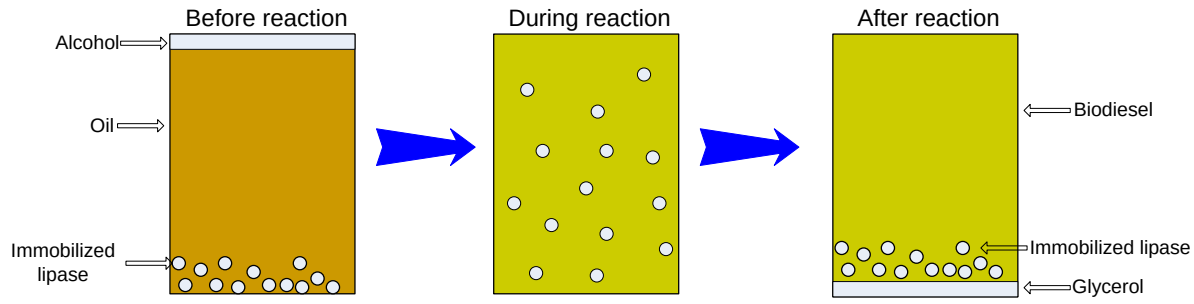

Figure 4.2 Multi-phasic reaction system of transesterification catalyzed by immobilized lipase

4.2.2 Inhibitory effect of excess ethanol

Compared to the studies about the effect of methanol on lipase, fewer studies about the effect of ethanol on the lipase have been reported and the findings of these works agree on that the ethanol can cause less deactivation to enzyme than methanol (Deng et al., 2005; Shimada et

al., 2002; Kumari et al., 2007). However, it was found by Shimada and coworkers (2002) that the immobilized lipase were less stable with the two-step process (1/3 eq for the first step and 2/3 eq on the second step) than the three-step process (1/3 eq for each step). One of their explanations is the excess ethanol can cause lipase deactivation.

Experiments have also been conducted in this work to investigate the effect of ethanol. It was found that the effect of ethanol was associated with the lipase source and the water content in the ethanol, which will be further discussed in the following section of biocatalyst selection. It was also found that the excess 96% ethanol over the solubility limitation can inhibit the lipase but the effect was reversible, meaning the activity can be recovered when there is no more undissolved ethanol. One such example is given in Figure 4.3, which shows the lipase was inhibited at the beginning of the reaction by the large excess ethanol in one-step addition compared to the continuous addition and three-step addition (0.5 eq at 0, 2 and 4 hours). The inhibitory effect was relieved as the reaction carried on because the formation of DAG and MAG improved the solubility of ethanol.

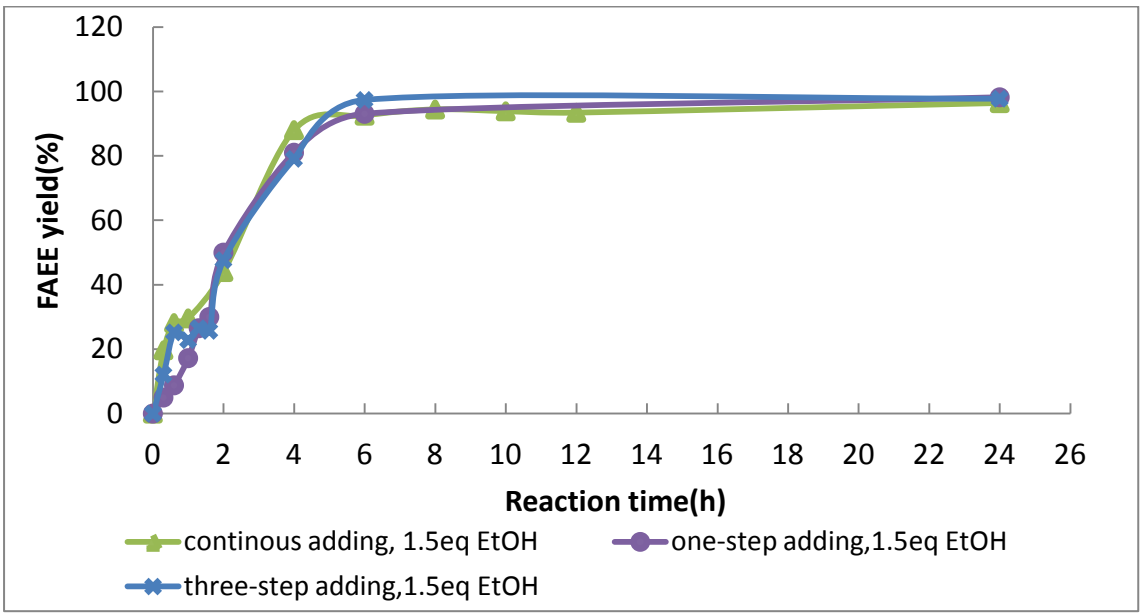

Figure 4.3 The effect of different mode of adding 1.5 eq 96% ethanol on the reaction progress Conditions: 5 wt% immobilized TLL (NS 88001) at 35 ^{o}C in an agitated reactor

4.2.3 Inhibitory effects of by-product glycerol

Glycerol has less effect on soluble alkaline catalyst in chemical process but it can cause an undesirable effect to immobilized lipase in enzymatic route. The effect is mostly a physical inhibition to biocatalyst when glycerol forms a hydrophilic layer outside the immobilized

lipases and thus reducing the diffusion of the hydrophobic substrate to the active site of the lipase. Thus, the operational stability of the biocatalyst can be greatly shortened and thereby the economic viability of the process will be influenced.

Such negative effects of glycerol have been systematically investigated with the aid of phase behavior test and study of reaction kinetics as presented in Paper 1 (Appendix D). A dyeing method has been developed to study the phase behavior by *in situ* visualizing the partitioning and accumulation of glycerol during the ethanolysis reaction to illustrate the interaction of glycerol with immobilized lipases.

As has been reported in Paper 1, the effect of glycerol is closely related to the hydrophobicity of the support of the immobilized lipase. Glycerol has a great affinity for hydrophilic supports, such as silica (the support of Lipozyme TL IM), little or no affinity for hydrophobic supports, like polymethylmethacrylate (the support of N435 and NS 88001). The results are shown in Figure 4.4.

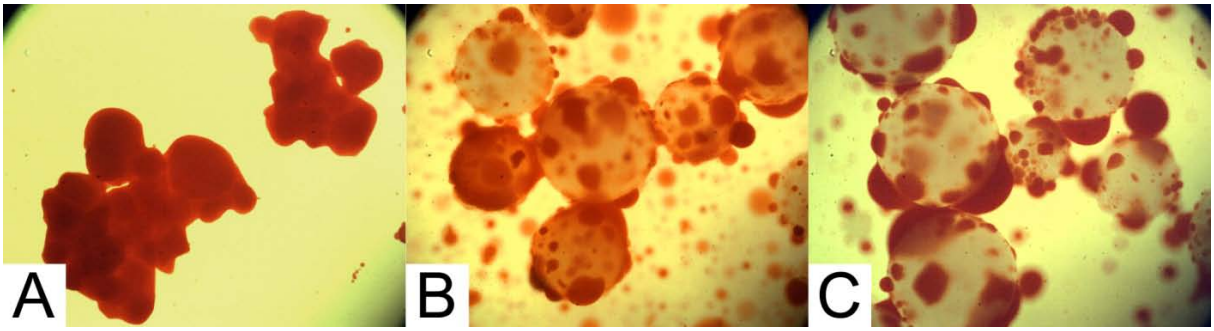

Figure 4.4 Glycerol partitioning in ethanolysis catalyzed by different immobilized catalysts. Lipozyme TL IM (A), N435 (B) and NS 88001 (C)

4.3 Evaluation and selection of biocatalyst

The immobilized lipase is the chosen formulation of the lipase in this work due to some of its advantages which can possibly benefit the process. Compared to the liquid formulation of lipase, the immobilized formulation of lipase can avoid the contaminant of residual enzyme in products and extend the life time of the enzyme. For the transesterifiaction, the separation of glycerol is much simplified in immobilized lipase catalyzed reactions and consequently the purity of glycerol is also much higher. The saved cost from purifying crude glycerol can help the economical viability of enzymatic biodiesel production.

4.3.1 The selection of lipase

Extracellular lipases from various microorganisms have been widely immobilized for biodiesel reactions including *Candida antartica*, *Pseudomonas fluorescens*, *Pseudomonas cepacia*, *Burkholderia cepacia*, *Rhizomucor miehei*, *Mucor miehi*, *Chromobactrium viscosum*, *Porcine pancreas*, and *Thermomyces lanuginosus* (Nelson et al., 1996; Iso et al., 2001; Shah et al., 2006; Orçaire et al., 2006; Demirkol et al., 2006; Shah et al., 2004; Yesiloglu, 2004; Watanabe et al., 2007b; Yagiz et al., 2007). A few of them have been commercialized, such as Novozym 435 (N435), Lipozyme RM IM and Lipozyme TL IM, among which the most commonly used immobilized enzyme for making biodiesel is N435 (Al-Zuhair, 2007).

However, 96% ethanol is not preferred by N435 (Deng et al., 2005). It was found in this work that the TLL showed both high activity and stability with 96% ethanol (results are presented in Paper 2 of Appendix D). Therefore, the selection of biocatalyst needs to focus on immobilized TLL.

4.3.2 The selection of carrier

As the by-product glycerol can be problematic to the immobilized lipase, the dyeing method has been applied to screen the carriers for lipase immobilization according to their interaction with glycerol. Glycerol was found to have great affinity for silica, less for polystyrene and no affinity for carriers made from polymethylmethacrylate and polypropylene. It was also found that the immobilization of enzyme on the support influenced the adsorption of glycerol to the surface of the enzyme carrier. The details of findings can be found in Paper 1.

To avoid the mass transfer problems caused by glycerol adsorption, the ideal carrier should be hydrophobic enough and therefore TLL should be immobilized on a hydrophobic carrier, such as polymethylmethacrylate.

The carriers of the immobilized lipase need to be sufficiently rigid and incompressible so that they can stand the mechanical agitation or high pressure in the reactors. Such evaluations have been conducted within reactor evaluation in the following section.

To sum up, NS 88001 (TLL immobilized on a hydrophobic carrier) meets all the requirements and has been selected to carry out this reaction.

4.4 Reactor evaluations

The multiphasic nature of the immobilized lipase-catalyzed transesterification defined in the previous step determines the reactor candidates should be heterogeneous reactors. This section evaluates reactor candidates (STR and PBR) with respect to the performance of NS 88001 selected from previous step. The other purpose of this section is to identify the key operational constraints that influence the process efficiency.

4.4.1 STR

Most reported immobilized lipase-catalyzed biodiesel studies have been so far carried out in shaking flasks of modest scale and have been focused on the effects of concentrations of enzyme, alcohol and water with a variety of oils containing differing amounts of FFAs. Reports are really scarce in the literature regarding the enzymatic production of biodiesel from an actual reactor sense.

In this work a lab-scale STR of a working volume of 150 mL has been used to evaluate the enzymatic transesterification within a power input per volume of 1.0 W/L which is close to industrial application. The reactor is equipped with a marine propeller and four wall baffles, which is illustrated in Figure 4.5.

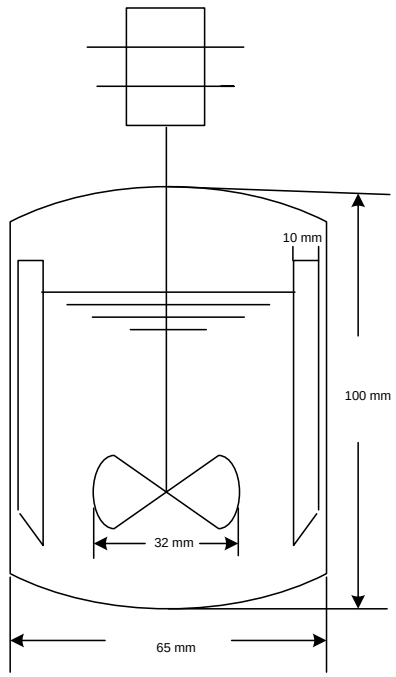

Figure 4.5 Configuration of a lab-scale STR

4.4.1.1 Mass transfer through multiple phases

The efficient mixing provided by an STR is useful for adequate dispersion of typical multi-phasic reaction systems catalyzed by immobilized lipases, especially those involving high viscous reactants. As presented in Paper 2, the mixing at just suspended speed (Njs) was sufficient for the mass transfer requirement at the beginning of the reaction, which was corresponding to a low power input (0.2 W/L). The mass transfer cannot be significantly improved by increasing the stirring speed above Njs. The formation of viscous glycerol during the reaction requires higher mixing intensity, 0.5 W/L required when absolute ethanol was used without enzyme deactivation by excess ethanol.

4.4.1.2 Integration of SFS to STR for solving inhibitory effect of excess ethanol

To avoid the inhibitory effect of excess ethanol, SFSs have been proposed and verified in an STR. Stepwise addition of alcohol has been widely used in lab-scale studies of enzymatic transesterification, especially methanolysis (Watanabe et al., 2000; Nie et al., 2006; Sanchez and Vasudevan, 2006). Continuously feeding alcohol to match its consumption rate can maintain an optimal and constant concentration in the reaction to keep lipase active and stable, which has been rarely reported.

Both strategies have been studied in STR and presented in Paper 2 of Appendix D. High biodiesel yields (˜95%) and good stabilities of NS 88001 (no apparent activity loss for 5 batches of reuse) with 1.5 eq 96% ethanol have been achieved with these operations.

4.4.1.3 Mechanical stability of NS 88001 in STR

Mechanical stability of NS 88001 has also been investigated in the lab-scale STR with a testing period of 120 hours. Above 80% particles were found intact at a P/V close to an industrial application (1.0 W/L). The shear damage to the catalyst particles increased as the increase of the power input.

NS 88001 particles at a higher catalyst loading (5%) were subject to slightly more severe mechanical damage than a lower catalyst loading (2.5%) due to the more collision at a higher density of particles.

NS 88001 particles were found less mechanically stable in the actual reaction mixture than they were in biodiesel or a mixture of oil and glycerol (2:1 w/w), which was probably due to the varied turbulence intensities through the reaction progress. Additionally, the carrier

material of NS 88001 is probably not stable with the reactant ethanol, which makes the carrier more vulnerable to the shear stress in the reaction mixture.

4.4.1.4 Operation modes of STR

Batch and continuous operations have been evaluated in Paper 2 and Paper 4 of Appendix D. There has not been any published work about enzymatic biodiesel production in CSTR, which makes the work presented in Paper 4 about CSTR novel. The results are compared in Table 4.1.

BSTR has the best productivity based on one batch without considering the downtime. The immobilized lipase can be easily separated from products and retained in the reactor so that preparing the catalyst for each batch can be saved from downtime. However, the downtime is still inevitable and will result in a low overall space-time-yield of BSTR. Furthermore, the solutions are limited for dealing with activity degradation of immobilized enzymes as the increasing time of reuse, either adding more fresh biocatalyst to compensate the deactivated enzyme or changing the reaction conditions (increasing residence time or temperature). But these solutions are restricted by the reactor volume or the production capacity (Nielsen et al., 2008). Therefore, batch operation of STR is not optimal to the industrial enzymatic biodiesel production.

Table 4.1 Evaluation of batch and continuous operations of STR

Operation	FAEE yield[a]	Residence time[b]	Productivity[c]	Paper index
Batch (BSTR)	95	6	3.18	2
Continuous (2 CSTRs)	76	4.9	3.1	4
Continuous (3 CSTRs)	85	9.5	2.36	4

a. unit: wt%; b. unit: h; c. unit: kg FAEE(kg enzyme)$^{-1}$h^{-1}

Continuous operation of STR offers the possibility of a stable process, which simplifies process control and facilitating efficient use of manpower, making it an attractive alternative to the traditional batch processes. However, the volumetric efficiency of one CSTR is low but the use of CSTRs in series can improve the net volumetric efficiency of the process. As Table 4.1 shows, the efficiency of 2 CSTRs is similar to BSTR. But it is very difficult to operate a

CSTR system to achieve equilibrium conversions. It requires a large residence time to reach high conversions as the process option of 3 CSTRs in Table 4.1 shows and consequently it lowers the process efficiency.

An STR can provide sufficient mixing to avoid biocatalyst clogging by glycerol, which means less or no need for a glycerol separation step between tanks in CSTRs. Therefore, another advantage of CSTRs is that the loss of alcohol can be avoided without intermittent removal of glycerol because alcohol and glycerol are highly miscible.

4.4.2 PBR

Some modification of the configurations are needed for conventional STRs to retain the biocatalyst in the reactors which may make it more convenient to use a PBR, which explains why most continuous biodiesel productions use immobilized lipase have been carried out in PBRs, although at a small scale (Halim et al., 2009; Watanabe et al., 2000, Shaw et al., 2008; Hsu et al., 2004; Nie et al., 2006).

As illustrated in Figure 4.6, a lab-scale PBR set up has been used for studying NS 88001-catalyzed transesterification and the evaluation results are presented in Paper 3 of Appendix D. Transesterification was carried out in three steps and 0.5 eq 96% ethanol was added on each step to avoid any inhibition by undissolved ethanol. The reaction mixture was repeatedly passed through a single column to simulate the effects of continuous production in a series of columns. To diminish the negative effect of glycerol, intermittent removal of glycerol was carried out between passes (virtual columns). 92.8% FAEE was obtained after the reaction mixture experienced 20 passes through the column at the flow velocity 7.6 cm min^{-1}, equivalent to passing the reaction mixture through 20 identical columns containing the same amount of NS 88001. The loss of ethanol in the glycerol removal can probably explain the lower yield than that in the BSTR.

The efficiency of this process has been compared to published work on immobilized lipase-catalyzed transesterification in Table 4.2.

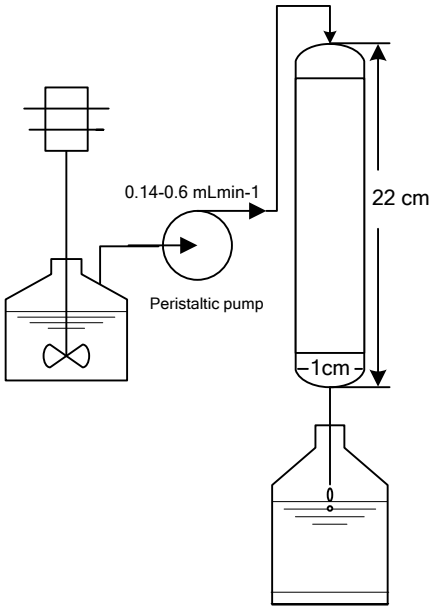

Figure 4.6 Lab-scale PBR set up

N435 has been mostly applied in PBR study for biodiesel probably because it is one of the few commercially available immobilized lipases and its hydrophobic carrier can have less mass transfer problems related to by-product glycerol.

Many studies on immobilized lipase-catalyzed biodiesel production in a PBR involve the use of a co-solvent to reduce the viscosity of the reaction mixture, enhance the solubility of alcohol in the feedstock and dissolve glycerol, improving the mass transfer and allowing operation within a single liquid phase. The efficiencies of PBRs are thusly improved by using these co-solvents, as the studies carried out by Shaw et al. and Royon et al. shown in Table 4.2.

Instead of using co-solvents, relatively higher flow velocities are usually applied in solvent-free systems to obtain sufficient mass transfer for the reactions, except the work by Watanabe and coworkers, who used an extremely slow flow velocity to obtain a high FAME yield (93%) but ended up with a very low productivity. The productivity of NS 88001, presented in Paper 3 is higher than those achieved by Hama et al. and Watanabe et al. using N435 and methanol, which indicates that NS 88001 is a promising catalyst candidate for ethanolysis. Additionally, the efficiency of the process developed in the PBR of this work is close to that of BSTR under similar conditions (Table 4.1).

Table 4.2 Comparison of process efficiency of immobilized lipase-catalyzed biodiesel productions in PBR

Catalyst	Alcohol	Total alcohol[a]	Solvent	Flow velocity[b]	Productivity[c]	Reference
NS 88001	96%EtOH	1.5	free	7.6	2.52	Paper 3
N435	MeOH	1.67	free	9.3	1.38	Hama et al., 2011
N435	MeOH	1	free	0.06	0.6	Watanabe et al., 2000
N435	MeOH	1.43	n-hexane + t-butanol	2.03	3.96	Shaw et al., 2008
N435	MeOH	2	t-butanol	0.57	4.02	Royon et al., 2007

a. unit: eq; b. unit: cm min^{-1} ; c. unit: kg FAEE (kg enzyme)$^{-1}$ h^{-1}

The maximum flow velocity 7.6 cm/min tested in the lab-scale PBR gave a pressure drop of about 1.1 bar, which is lower than the maximum pressure drop, 3 bar provided by catalyst supplier for the sake of safety and a stable performance of NS 88001. For industrial practice, this specification for pressured drop limits the column length as the linear velocity is maintained for scaling up and pressure drop increases linearly as the length. It consequently affects the number of PBRs in the process for achieving the same conversion.

4.4.3 Operational windows for reactors

Bioprocesses are normally complex and 'fragile' owing to the complicated mechanism of enzyme and the sensitivity of enzyme to the reaction conditions. Therefore, knowing a safe zone of dealing with enzymes can benefit enzyme users by giving a robust bioprocess. This can be achieved by a useful tool named 'operating window', which is an operating space determined by constraints and correlations of a process. This two dimensional graphical window can visualize the process complications, identify the key elements to the efficiency of a process and guide the process design and optimization (Woodley and Tichener-Hooker, 1996).

The tool has been applied in examples of STR and PBR in the studied case of immobilized lipase catalyzed transesterification and the two operational windows are presented in Figure

4.6, respectively. Strategies to enlarge the operating space (window) are also given in Paper 2 and 3 since the larger window allows more flexible operation and reduces the chance of operating mistakes.

A comprehensive understanding of the reaction system and reactor properties is a prerequisite for choosing variables and constraints and finding their correlations to build up such operational windows. For example, the reaction rate is mostly dependent on the enzyme activity and mass transfer of substrates by mixing in STR, which relate to the catalyst loading and power input. Therefore, they are chosen as the variables in the operational window. The intention for industrial implementation of the studied process sets constraints for energy input and biocatalyst productivity. The glycerol associated mass transfer problem sets another constraint of minimum stirring speed/power input. The damage to immobilized enzyme particles is the correlated effect from particle collision and shear stress from the impeller, which are related to the catalyst loading and power input. The operational window is thus formed by constraints for these variables and their correlation.

Similarly, in a PBR system the superficial velocity affects the mass transfer and varies as the cross-section area of the reactor at a certain volumetric flow rate. Therefore, the superficial velocity and the cross-section area are the key variables to frame the area for defining the window. The productivity of biocatalyst determined by the industrial application sets one constraint of minimum oil conversion of each pass through PBR; the choice of immobilized lipase sets another constraint of maximum pressure drop. The constraint of mass transfer limitation is set by the minimum superficial velocity.

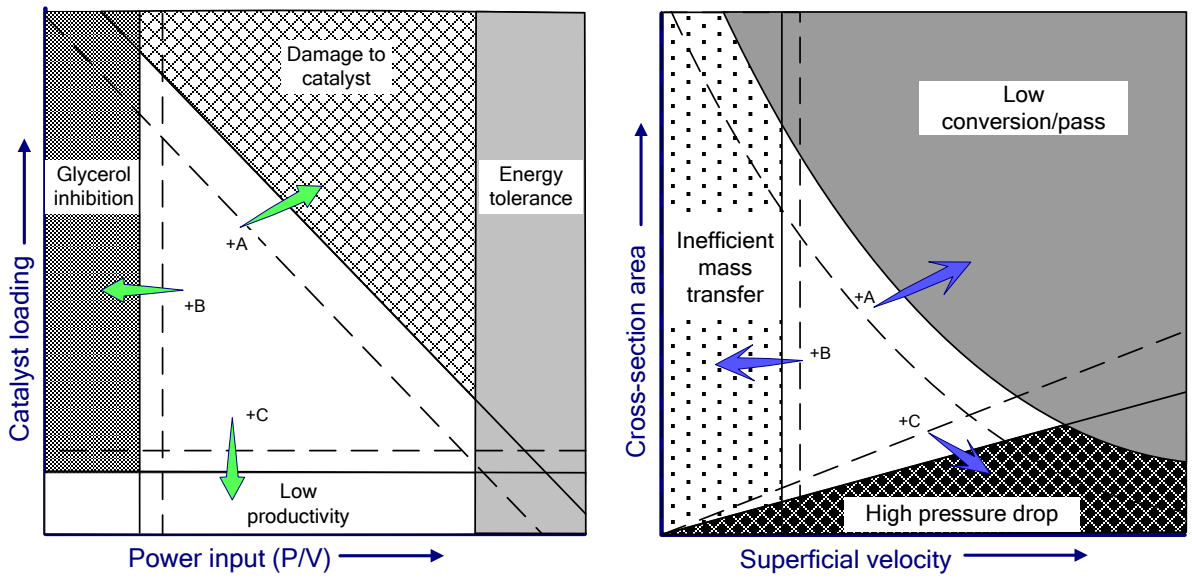

Figure 4.7 Operating window for STR (left) and PBR (right) (A, B, C and D illustrate different expansions of the window, + indicates the direction as the arrow shows; details can be found in Paper 2 and 3)

Figure 4.7 shows two conceptual windows while the window can be defined with quantitative relationships between constraints and variables to provide more specific operating guidance. Such a quantitative window of STR is given discussed in Paper 2 and the operational limits identified by this approach (e.g. the mass transfer limitation or the mechanical damage to the biocatalyst) can aid the scale-up of this process, which are addressed in the following section.

4.5 Pilot plant validation of BSTR process

4.5.1 Introduction to the pilot plant validation

After the lab-scale evaluations, pilot scale experiments are usually needed to validate the established small-scale processes. The purpose of having the pilot-scale process is to test the biocatalyst performance at a larger scale which can be probably affected by the fluid phenomena that are not presented in lab-scale experiments. The pilot plant is also a foundation or platform for building up the process control system. Even though, pilot plant is sometimes skipped in the scaling up process since it is still a costly apparatus (Donati and Paludetto, 1997).

4.5.2 Scale-up procedure

For the NS 88001-catalzyed transesterification system, the mass transfer across the multiple phases has been identified as a critical operational limit of the process. It is therefore necessary to maintain the same turbulence (constant Re) but T_m will be greatly increased, related to the square of the scale-up factor. Therefore, another approach needs to be taken for the scale-up procedure, which is the similar geometry and constant power input (P/V) as the lab-scale set up.

It is recommended for multiphasic reactions that two or more different scales are necessary through the scaling-up procedure and the vessel size diameter is varied by at least a factor of 2 (Paul et al., 2003). Therefore, a larger scale-up factor of approx. 4 was used in this work, as shown in Table 4.3 which details the reactor configurations at both scales.

Table 4.3 Configurations of lab-scale and pilot-scale STRs

Dimensions (mm)	Lab-scale	Pilot-scale
Reactor diameter (T)	65	250
Reactor height (H)	100	477.66
Propeller diameter (D)	32	151.5
D/T	0.5	0.6
Propeller position to bottom (C)	15.4	70
C/T	0.24	0.28
Baffle width	10	25
Material	Borosilicate glass	Stainless steel-316
Working volume (L)	0.15	10

The pilot process layout is shown in Figure C2 in Appendix C. There is one substrate tank of oil which is delivered to reactors by gravity and two other substrate tanks of ethanol (96% and absolute, respectively), delivered to reactors by gear pumps. Transesterification and polishing (esterification) reactions take place respectively in two identical 20 L STRs. A flash unit is in place connected to a vacuum pump for evaporating ethanol and water, which are recovered in a vessel. The flash unit is also used as a settling tank for glycerol separated from biodiesel product.

4.5.3 Validation conclusions

The experimental results of the pilot scale reactor are presented in Paper 2, which shows that the reaction performance in the pilot-scale STR correlates to that in the lab-scale STR to a satisfactory extent in terms of initial rates, final yields and reaction profiles, although the initial rates obtained from pilot-scale STR are a little lower than those from lab-scale STR.

High stability of NS 88001 with 96% ethanol was also observed in the pilot-scale STR with a little activity loss through four repeated use in terms of both final FAEE yields after 6 hours and initial rates without any washing to the catalyst between batches. It indicates that the scaled STR at studied conditions can provide a similar hydrodynamic condition as that presented in the lab-scale STR to maintain the stability of the lipase.

The increased shear stress in pilot-scale STR was observed from the mechanical stability results of the immobilized lipase (NS 88001). But the size distribution of broken particles was not found to clog the filter for retaining the catalyst in the reactor.

In short, the lab-scale STR process has been successfully validated in the pilot-scale STR indicating the sufficient flexibility and robustness of the process. It also means that the performance of NS 88001 in a scaled STR can be accurately predicted by that in a smaller STR.

4.6 Discussions

For the process development of enzymatic transesterification, there are some further factors which can potentially affect the process efficiency and they are discussed in this section.

4.6.1 Liquid formulation of lipase

Liquid formulation of lipase can benefit the process with a lower catalyst cost and a simplified phase condition in the reaction as addressed in section 1.3. The other advantages of using free lipase in a liquid formulation include a.) the negative effect of excess water found with immobilized lipases could be ignored in free lipase-catalyzed systems (Al-Zuhair, 2007); b.) by-product glycerol is less of a problem to liquid lipase than immobilized lipase since glycerol is normally added into liquid lipase formulation as enzyme stabilizer.

However, the high content of water in such a formulation can induce more hydrolysis and raise the level of FFA, making the product more difficult to meet the biodiesel specifications.

The separation of lipase from products is more difficult even though the lipase exists in the hydrophilic glycerol-rich phase, which gives rise to the risk of contaminating the products by the lipase. Membrane technology may be a solution by separating the product from the lipase. However, it is still a high investment and the prevention and elimination of membrane pore plugging is considerably difficult (Dubé et al., 2007; Balcáo et al., 1996).

Very few studies have been so far reported with the liquid formulation of lipase in biodiesel production. Two commercial soluble lipases NS 81006 and NS 81020 from genetically modified *Aspergillus niger* and *Aspergillus oryzae* have been used in the biphasic aqueous-oil methanolysis (Chen et al., 2008). It was found that NS 81006 was inhibited by high free fatty acid whereas NS 81020 worked effectively with oleic acid. It was expected that the combination use of the two soluble lipases for producing biodiesel from high-acid-value oils could be an alternative to the immobilized lipases.

4.6.2 Multi-lipase catalysis

Lipases of different catalytic abilities or in different forms can be combined in use to benefit the biodiesel processes with respect to process efficiency or process economy.

A mixture of immobilized lipases (Lipozyme TL IM and N435) was used in a single stage to improve the catalytic performance for a higher biodiesel yield (Li et al., 2006). And the price difference of the two immobilized lipases can also reduce the cost contribution of catalyst in this process.

Paper 3 also presents an example using multi-lipase in two separate stages. The proposed process applies NS 88001 and N435 separately, the first stage catalyzed by NS 88001 for transesterification to make most of the biodiesel product followed by N435-catalyzed esterification to convert FFA and partial glycerides (DAG and MAG) to biodiesel so that the finishing product can meet the biodiesel specifications. The multiple-lipase catalyzed process allows an efficient use of feedstock and reduces the energy consumption in product purification.

Another example of two-stage multi-enzymatic process was developed by Watanabe et al. for converting acid oil to biodiesel (Watanabe et al., 2007a). They used *Candida rugosa* lipase

which is in a liquid formulation of a lower price and has a high hydrolytic activity in the first step of hydrolysis. It was followed by the second step of esterification catalyzed by immobilized *C. antarctica* lipase, which catalyzes the esterification of FFA much faster than methanolysis of TAG. This combined use of free lipase and immobilized lipase can save the cost on biocatalyst.

4.6.3 Co-solvent

Due to the multi-phasic reaction system, many studies on immobilized lipase-catalyzed biodiesel production involve the use of a co-solvent, especially in PBRs where the mass transfer can be restricted. High process efficiency can be achieved in the co-solvent system as shown in Table 4.2, which can be explained by the following advantages of using co-solvent.

a.) Enhance the tolerance of lipase for alcohol and simplify the operation by increasing the solubility of alcohol in hydrophobic compounds.

b.) Accelerate the reaction rate by reducing the viscosity of the reaction mixture as well as dissolving the by-product glycerol resulting in an improved diffusion rate of substrate to the active site of lipase. It is extremely useful to a flow restricted reactor, such as PBR (Dossat et al., 1999; Royon et al., 2007; Shaw et al., 2008).

c.) Stabilize lipases by maintaining the required water activity for the conformational flexibility.

Hexane, propanol and tert-butanol are most often used organic solvents in the enzymatic biodiesel productions (Keng et al., 2008; Li et al., 2006; Nie et al., 2006).

However, compared to solvent-free system there are some drawbacks of using co-solvent for biodiesel production limiting the industrial application of solvents. Firstly, it makes the biodiesel production more costly because of the investment of reactors of large volume, the solvent recovery and the treatment of the organic waste; Secondly, it adds risk to the plant safety in that most organic solvents are volatile and flammable; and finally it is less environmental friendly (Fjerbaek et al., 2009; Nielsen et al.,2008).

4.6.4 Glycerol removal

The mass transfer limitation can be caused by glycerol clogging the immobilized lipases and this effect varies as the conditions, e.g. reactor choice and operation conditions. Table 4.4 lists some strategies for removing glycerol with their advantages as well as the limitations of applying them in the process.

Table 4.4 Comparison of glycerol removal strategies

Solution	Requirement	Advantage	Limitation
Add adsorbent	Higher affinity for glycerol than the catalyst	Material like silica is cheap	Increased volume; not for continuous operation; glycerol recovery
Change reaction medium	Dissolve glycerol	Simplified operating conditions	Increased volume, environmental considerations; Increased cost
Change carrier for enzyme immobilization	Less or no affinity for glycerol; Hydrophobic carrier	Improved characteristics; More adaptable	Expensive
Increase shear at catalyst surface	Sufficient	Broad applicability	Power input; damage to the catalyst; pressure drop

Among all strategies listed in the above table, the most adaptable and cheapest solution is the last one (increasing the shear at catalyst surface) because all reactors are capable to provide the mixing effect. However, one single strategy is often not enough in many cases to sufficiently remove the glycerol form the biocatalyst surface. Multiple strategies need to be combined to deliver a better effect of removing glycerol. For example, increasing the shear at the catalyst surface is only effective if the carrier is hydrophobic because it has been observed that the silica-based TL IM is still subject to glycerol clogging even in a vigorously agitated reactor.

4.6.5 Reactor combination

As identified in Paper 2 and 3, an STR allows much better phase contact than a PBR but the impeller of STR can cause more damage to immobilized enzyme particles than PBR, although both reactors can be managed to achieve similar efficiencies. Paper 4 also indicates that it is impractical to achieve high conversions in CSTRs due to the limited dilution rate.

Therefore, combining these reactors can possibly compensate the drawbacks of each other and maximize their advantages. Such a process is proposed and shown in Figure 4.7. It is composed of two CSTRs in series in conjunction with a CPBR for completing the transesterification reaction with a final conversion of 90 %. It can reach a process efficiency of 2.38 kgFAEE (kg enzyme)$^{-1}$ h^{-1}.

This process achieves a slightly higher productivity than the 3CSTRs (2.36 kgFAEE (kg enzyme)$^{-1}$ h^{-1}), which is because the process has not been optimized. It should improve more if PBR was introduced earlier in the reaction progress or push the conversion further in the PBR stage. Nevertheless, it is still promising to have such a process of combined reactors to obtain both high FAEE yield and process efficiency as well as the credits gained from a better mechanical stability of immobilized lipases and less energy input in PBR.

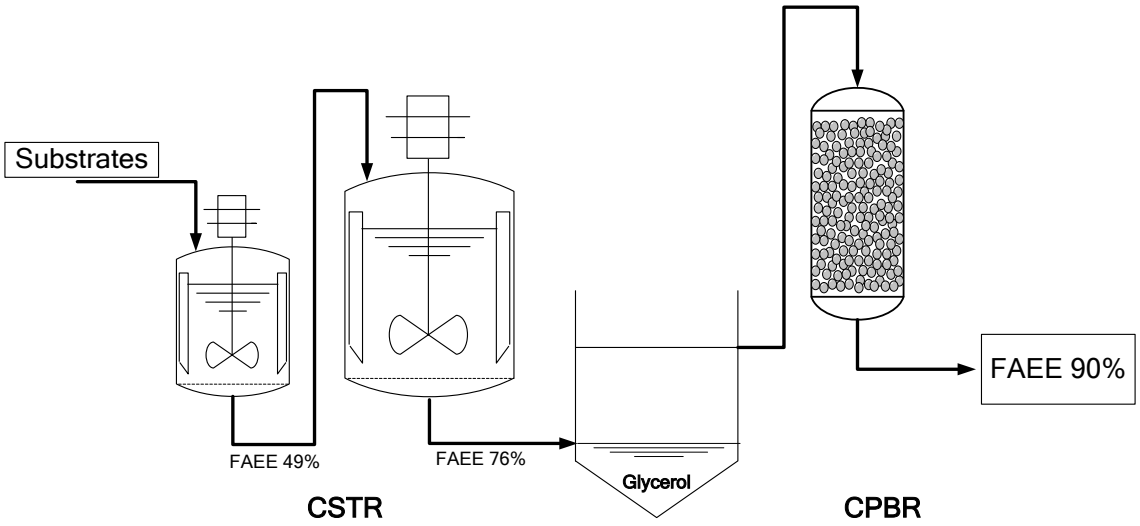

Figure 4.8 Illustration of process composed of CSTRs and CPBR

CHAPTER 5

Case study: immobilized lipase-catalyzed esterification for polishing biodiesel

5.1 Introduction

Although it is a big concern for biodiesel producers to meet specifications, most studies about biodiesel production are still focusing on reaction rate and biodiesel yield in a single transesterification stage and few studies have been published on making biodiesel meet product specifications (Hama et al., 2011). However, the biodiesel products from enzymatic transesterification are rarely within biodiesel specifications, particularly for FFA content, when using the 'wet' feedstock and 'wet' alcohol which is economically preferable since the water in the 'wet' alcohol can cause hydrolysis raising the level of FFA in the transesterification stage. Instead of applying energy-intensive distillation of biodiesel, an enzymatic polishing reaction (esterification) is proposed to simplify the purification and improve the biodiesel yield further.

Determined by the purposes, the reaction schemes are shown in Figure 5.1. The major reaction is the esterification of FFA and the other reactions include the transesterification of partial glycerides.

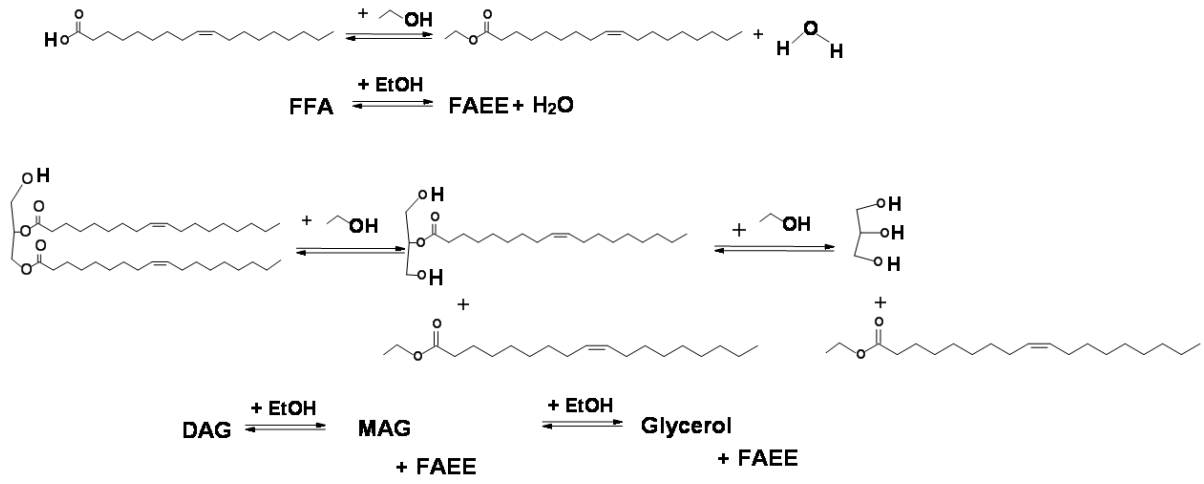

Figure 5.1 Reaction schemes of enzymatic esterification using ethanol

5.2 Key reaction characteristics

As an immobilized lipase-catalyzed reaction, the studied case is also a multi-phasic system. In contrast to the first case study, the reaction medium of esterification is less viscous and can dissolve a larger amount of ethanol due to the different composition of reactants, where the major component is FAEE and the rest of them are partial glycerides, FFA and water. Instead of glycerol, water is the major by-product of esterification and it cannot cause the same clogging effect on immobilized lipase as glycerol can do.

Therefore, the risks of mass transfer limitations and inhibitory effect of un-dissolved ethanol are much less in this case. Subsequently the process development for this reaction is also relatively easier with the knowledge gained from the first case study.

5.3 Process development

The major challenge of this process development is about overcoming the equilibrium limitation and pushing as much FFA to be reacted as possible in order to reach below the specified level (0.25 wt%). One possible solution is to remove the water during the reaction to drive the reaction towards the favorite side and any external water should be excluded from the reaction system. The water content should be maintained lower than 500 ppm for the studied system to achieve the target, which is kindly provided by a collaborative work. The other solution can be using a large amount of ethanol to push the reaction. These requirements determine the choice of lipase and the type of ethanol and loading.

It is mentioned in section 1.2 that N435 works much faster with smaller molecules like FFA, MAG and DAG than TAG. Furthermore, N435 works efficiently under very low water activity. Therefore, N435 is competent to carry out this reaction.

To avoid the external water, the ethanol used for this step has to be anhydrous, that is, absolute ethanol and the substrate for this step needs to be dried before contacting N435. As water is produced during the reaction, water needs to be removed along the reaction progress, which implies that the process should be either multi-step reactions with water removal between steps or instantaneous/*in-situ* water removing integrated reaction. The former one has been studied in a lab-scale PBR as presented in Paper 3. The selection progress for the process development is shown in Figure 5.2.

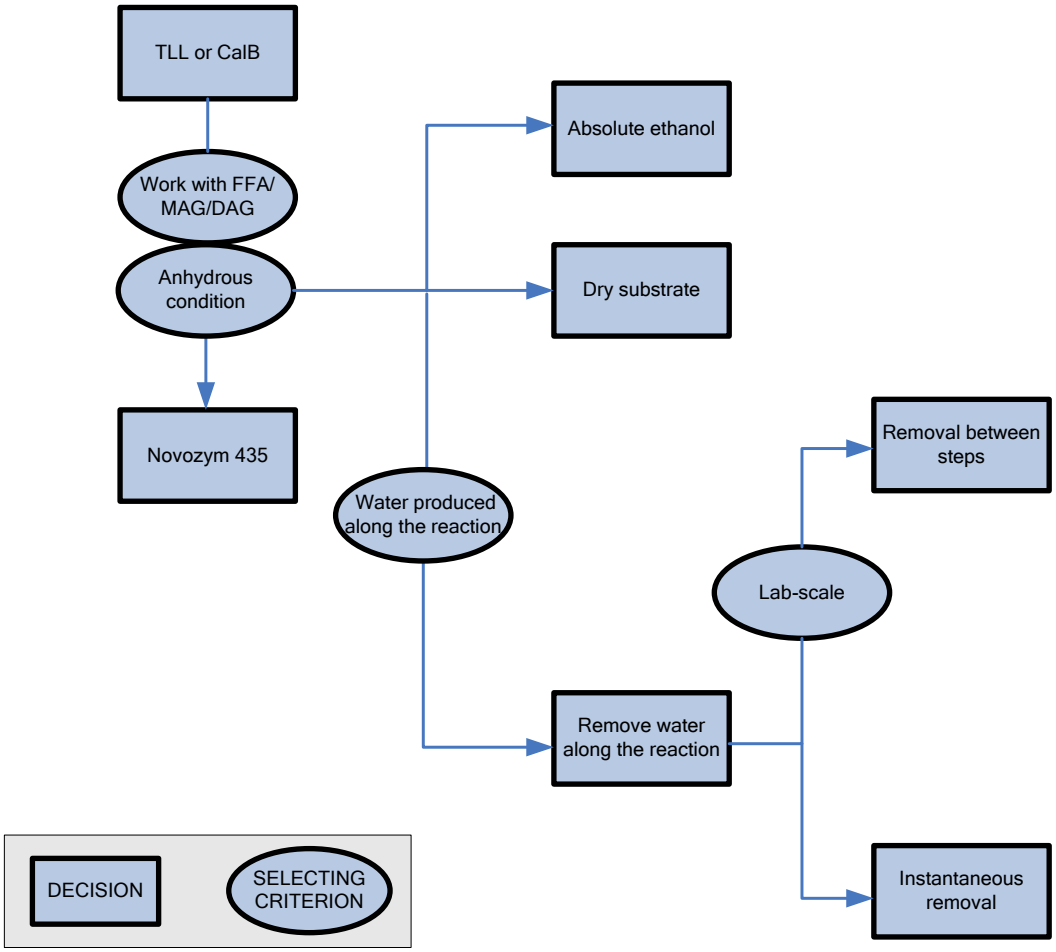

Figure 5.2 The selection progress for establishing the enzymatic polishing process

5.4 Conclusions and discussions

5.4.1 Conclusions of a lab-scale PBR process

N435-catalyzed stepwise esterification for polishing biodiesel product has been carried out in a PBR in the lab and vacuum drying has been applied for drying lipid mixtures between steps.

Ethanol loading is one of the major focuses of this study. It has been found that a large excess of ethanol is need to push the reaction towards FAEE formation particularly when FAEE is already the major component of the reaction mixture. The tested ethanol loadings on each step (0.3-1.2 eq to original FA in the feedstock corresponding to a molar ratio to initial FFA from 14 to 57) did not significantly affect the steady-state FFA content of the step.

Vacuum drying is a useful method to remove water from the reaction mixture between steps but it is not selective as ethanol is also removed meanwhile.

It has been managed in the lab-scale set up to polish the biodiesel product and improve the biodiesel yield by converting FFA and partial glycerides to FAEE via four steps consuming a total amount of 1.2 eq absolute ethanol to the original FA in the feedstock. The comparisons of product compositions after two reactions with biodiesel standards are shown in Table 5.1.

Table 5.1 Product compositions after each stage (wt%)

Parameter	EN14214	After transesterification	After esterification
FAEE	>96.5	95.0	99.4
MAG	<0.8	1.25	0.10
DAG	<0.2	1.41	0.12
TAG	<0.2	0.64	0.14
FFA	<0.25	1.71	0.24

5.4.2 Discussions

5.4.2.1 Lab-scale and pilot-scale STR processes

Esterification in a lab-scale STR (1 L glass reactor) has been carried out by a project partner. The substrate composition and reaction conditions are similar to those of the lab-scale PBR in this work except the drying conditions. The intermittent drying was taking place inside the STR with the presence of enzyme, meaning the reaction continued during the drying process. As a result, the efficiency of this process was improved. The FFA content has been pulled down below 0.25% by 3 steps consuming a total amount of 1.0 eq absolute ethanol to the original FA in the feedstock. However, partial glycerides (DAG and MAG) and TAG in the biodiesel product were still beyond the specified levels.

The lab-scale STR process has been verified in a pilot-scale STR (10 L) identical to the pilot-scale STR for transesterification process. Both ‘in-spot’ vacuum drying (inside reactor with enzyme) and ‘off- spot’ vacuum drying (outside reactor) were tested and other conditions kept constant. FFA contents were successfully reduced below 0.25% in both drying ways. However, a further efficiency comparison of two drying ways is not possible due to a lack of data through the processes. However, the compositions of other glycerides were also ‘out of spec’, in agreement with lab-scale results.

In summary, the three-step esterification process in both lab- and pilot-scale STRs succeeded to bring FFA content to specifications but failed to make the glycerides also 'in-spec'. It is worthwhile to try more steps or use other means to get rid of the excess glycerides.

5.4.2.2 Water removal

As a by-product of esterification, water is preferably removed to shift the equilibrium resulting in a higher conversion. Although a simple but efficient solution of vacuum drying is used as an intermittent separation method in this work, some other lab-scale strategies have been found in literatures for selectively removing water *in situ*, as shown in Table 5.2. The advantages and limitations of them are also included.

Table 5.2 Comparison of strategies for water removal (Wang et al., 2006; Gubicza et al., 2000; Kwon et al., 1995)

Strategy	Solution	Advantage	Limitations
Adsorption	Silica gel or 3Å molecular sieve	Cheap, *in-situ* separation	Dehydrate enzyme Difficult to reuse
Evaporation	Heteroazeotropic distillation (n-pentane and water)	Mild condition (boiling point close to the reaction temperature); *in-situ* separation, continuous operation	Solvent issue, difficult to scale up
Membrane separation & evaporation	Pervaporation	*in-situ* separation; continuous operation; easy scale-up;	Higher cost; membrane fouling

5.4.2.3 Scale up

For the industrial application of this step, the major difficulty of scaling up is to remove water and integrate it to the existing system. The feasible and affordable solutions can be molecular sieve columns or falling film evaporators between reactors, the former has the advantage of no need for adding ethanol between reactors but the regeneration of the molecular sieves can be troublesome; the latter can be efficient on removing water as well as ethanol, which means ethanol needs to be added between reactors and it also requires more investment on equipments.

The final decision for adopting this polishing step into the biodiesel process is the trade-off of the saved cost from biodiesel distillation and improved biodiesel yield against the investment on the second enzyme and extra equipments.

CHAPTER 6

Industrial process evaluations

The previous chapters have addressed the technical and scientific challenges of the process development for immobilized lipase-catalyzed reactions. It is of course important to evaluate its economic and environmental feasibility, which is suggested in the methodology. This chapter performs process evaluation for six processes composed of transesterification and product purification for making 'in-spec' biodiesel with respect to material and energy balance and the conventional chemical process is taken as a bench mark for comparison.

The evaluations of mass and energy for each process are based on data collected from literature, unpublished work and reasonable assumptions. The process evaluation focuses on the reaction process and the following purification process to meet the biodiesel specifications of Europe (EN14214) in a scenario of an industrial scale.

Process 1 and 2 have been well reported and they are the conventional chemical process and the immobilized lipase-catalyzed methanolysis, both of which use conventional product purification characterized by FAME distillation. Process 3 has almost the same elements as the transesterification part of Process 2 except the absence of purification process due to the promising results obtained right after transesterification, that is, all product components within biodiesel specifications. Process 4, 5 and 6 are rarely studied but they show great potentials of sustainability by using lipase, ethanol and waste oil, under which circumstance the enzymatic polishing of product is also evaluated. All selected processes are placed in an order of ascending sustainability, as shown in Figure 6.1.

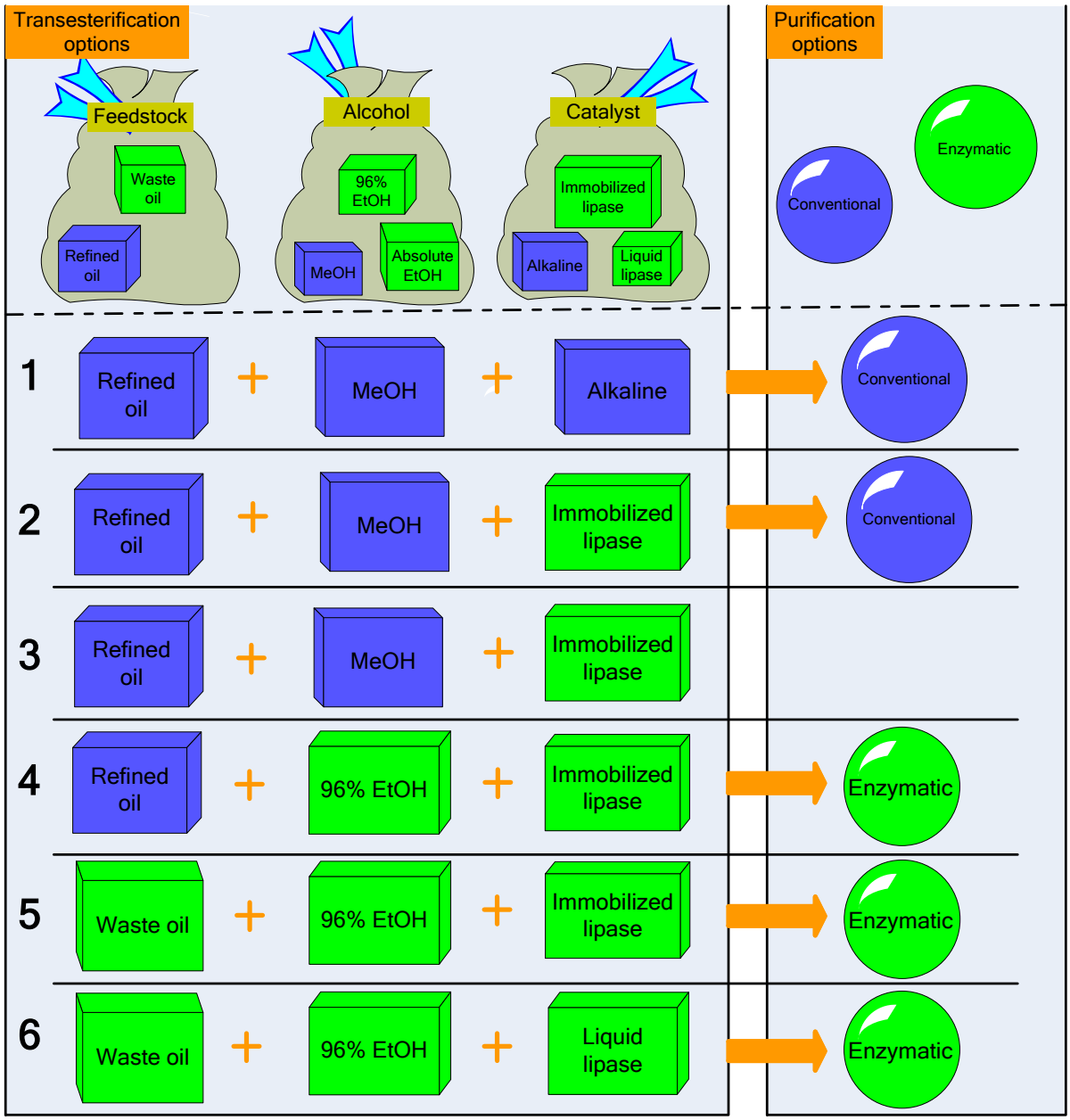

Figure 6.1 Process options for evaluations

6.1 Process conditions and assumptions for evaluation

Table 6.1 gives the process conditions of the selected processes and also specifies the source of data. The catalyst productivity of alkaline in process 1 is based on a single batch use. Without considering the deviation caused by the choice of reactor and operation difference, the biocatalyst productivity with methanol in process 2 and 3 is chosen the best value among available literatures of the same catalyst applied in the similar process, calculated based on 4 % catalyst loading, 54 cycle of reuse and 95% FAME (Shimada et al, 1999). However, the literature about the biocatalyst (NS 88001) productivity with ethanol is seldom. Process 4 and

5 adopt the productivity data (4800 kg FAME/kg enzyme) of the similar biocatalyst working with methanol in t-butanol (Li et al., 2006). This is based on the assumption that lifetimes of the lipase in both conditions are similar as the ethanol is reported to be less toxic to lipase and the t-butanol used as the co-solvent can diminish the methanol inhibition to lipase. In fact, the value (4800 kg FAME/kg enzyme) is very close to the expectation of the catalyst supplier to NS 88001 based on the current biodiesel market. The productivity data (0.8 ton biodiesel/kg enzyme) of liquid lipase for process 6 is a required value according to the catalyst price.

Process conditions for the enzymatic polishing step are given in Table 6.2. The mass balance and energy consumption of this stage are taken into account of the overall mass and energy balance of the whole process.

Table 6.1 Process conditions for biodiesel production

Process index	1	2	3	4	5	6
Major data source	Zhang et al., 2003; Sotoft et al., 2010	Watanabe et al., 2002	Hama et al., 2011	Paper 3	Paper 3 + assumptions	Pedersen, 2011 + assumptions
Feedstock	Refined rapeseed oil	Refined soy oil	Refined mixed soy and rapeseed oil	Refined rapeseed oil	Waste oil	Waste oil
Alcohol in transesterifi-cation	MeOH	MeOH	MeOH	96%EtOH	96%EtOH	96%EtOH
Alcohol to oil molar ratio	6:1	3:1	4:1	4.5:1	4.5:1	4.5:1
Catalyst	NaOH	N435	N435	NS 88001	NS 88001	Liquid lipase
Catalyst loading[a]	1	4	12	5	5	0.5
Catalyst productivity[b]	100	1200 (Shimada et al.,1999)	1200 (Shimada et al.,1999)	4800 (Li et al., 2006)	4800 (Li et al., 2006)	800
Oil conversion[c]	95.6	93.8	98.6	95	95	90
Reaction temperature (^{o}C)	60	30	30	35	35	35
Reaction time(h)	1	48	14.2	6	6	24
Necessary distillation	Water, alcohol and FAME	Alcohol and FAME	Alcohol	Water and alcohol	Water and alcohol	Water and alcohol
Enzymatic polishing	No	No	No	Yes	Yes	Yes
Improved oil conversion (mole %)				99.4	99.4	99.4
Reaction time				3	3	5

[a] unit in wt%; [b] unit in kg biodiesel/kg catalyst; [c] unit in mole %; highlighted part is the enzymatic polishing stage and more details are in Table 6.2

Table 6.2 Process conditions for enzymatic polishing step

Catalyst	Catalyst loading (wt%)	Alcohol	Alcohol to oil molar ratio for each step	Reaction time for each step (h)	Drying strategy
N435	5	Absolute EtOH	1:1	1	Vacuum drying (1 h) between steps

To facilitate the evaluations of the selected processes with respect to the mass and energy balances, some more assumptions are necessary to make and they are given as follows:

a. Average industrial scale of an annual production of 16000 tons biodiesel. According to Pruszko et al. 5 million gal per year (approx. 16000 tons per year) is a medium scale for a chemical biodiesel plant in America.
b. Refined oil: purely TAG; waste oil: TAG, 15% FFA and 0.2% water (Nielsen et al., 2008; Al-Zuhair, 2007)
c. The same activity of biocatalysts working on waste oil as on refined oil (Sanchez and Vasudevan, 2006, Watanabe et al., 2001), in other words, the same molar conversions from TAG to biodiesel in process 4, 5 and 6. The assumption is valid for both immobilized lipase and liquid lipase.
d. Batch operation in STR, energy consumption of which only considers agitation.
e. The same efficiency of alcohol recovery between ethanol and methanol, which is 94 % cited from a conventional chemical process (Harding et al, 2007 and Zhang et al., 2003).
f. 96% ethanol is removed as azeotrope by distillation.
g. Addition of 40 g water/g liquid lipase for activation of liquid lipase. Recycle use of water is not considered in mass balance.
h. Glycerol is separated from liquid lipase by the difference of physical property, such as molecule size; and the energy consumption for this procedure is not counted

6.2 Results of mass and energy evaluations

6.2.1 Mass evaluation

The material balances of all processes are illustrated in Figures 6.2-6.5, based on which the mass balance of each process is calculated and given in Table 6.2.

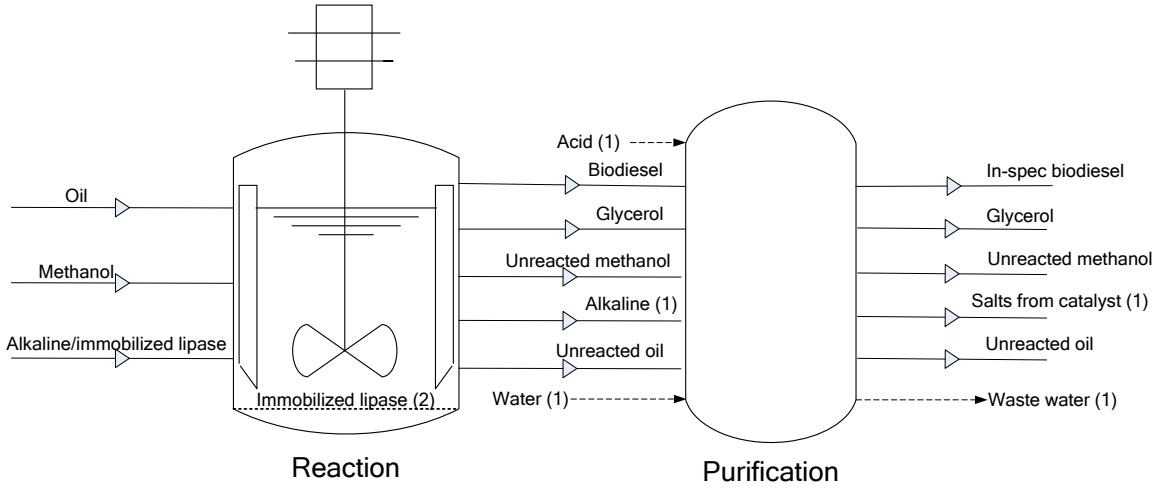

Figure 6.2 Mass balance illustration for process 1 and 2 with conventional purification

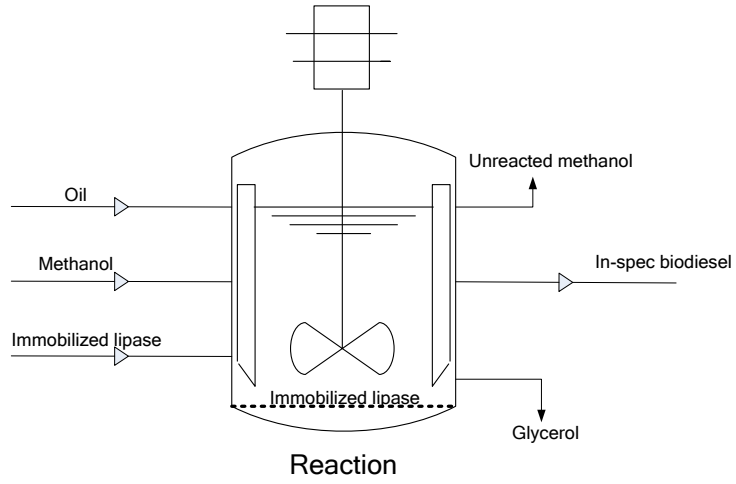

Figure 6.3 Mass balance illustration for process 3

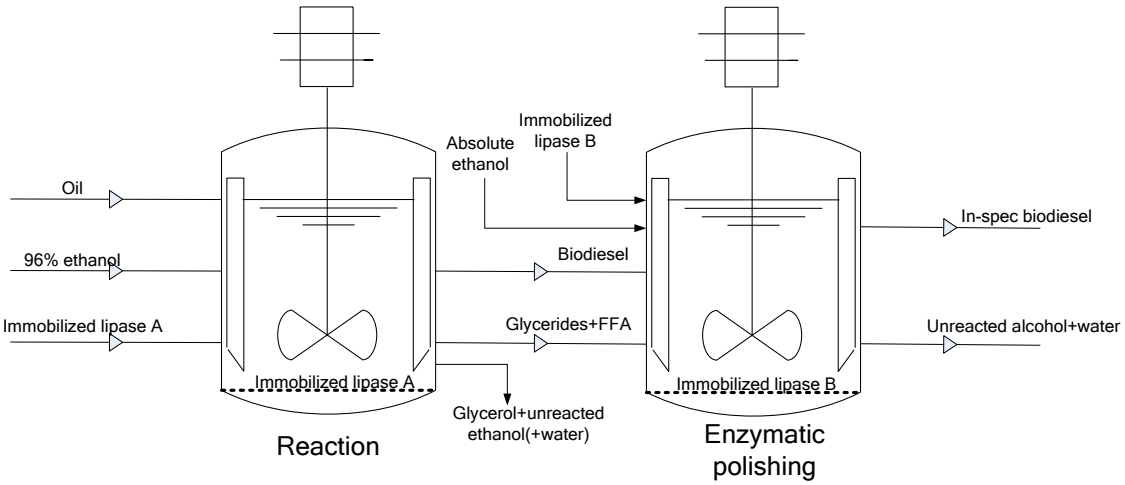

Figure 6.4 Mass balance illustration for process 4, 5 with enzymatic polishing

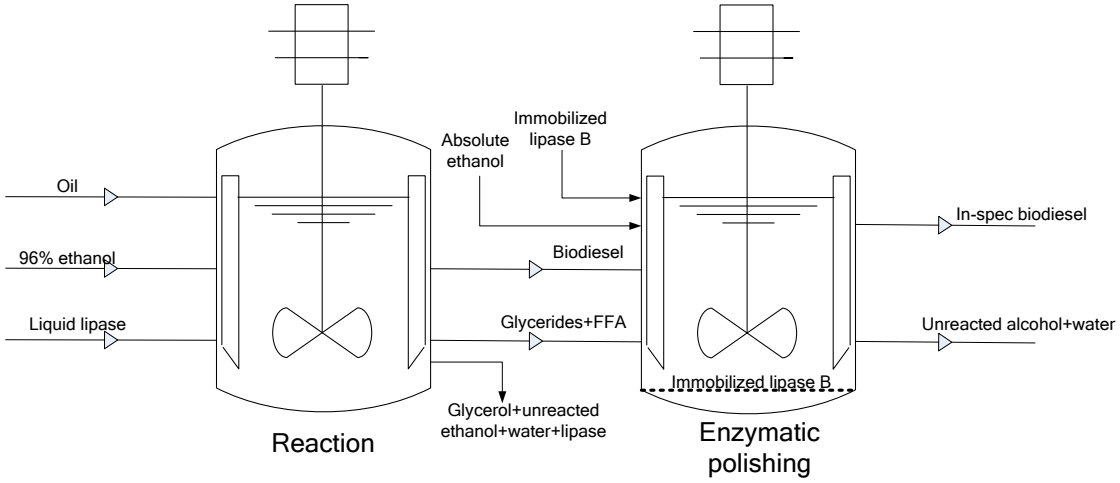

Figure 6.5 Mass balance illustration for process 6 with enzymatic polishing

Table 6.3 Mass balance of each process (unit in ton)

Process index	1	2	3	4	5	6
INPUT						
feedstock	16738.7	17057.6	15948.6	15330.1	15237.3	15237.3
alcohol	methanol	methanol	methanol	ethanol	ethanol	ethanol
	1791.7	1735.6	1705.8	2370.4	2370.4	2370.4
catalyst	159.4	13.3	13.3	4.0	4.0	20
water	7600					800
OUTPUT						
biodiesel (ton/year)	**16000**	**16000**	**16000**	**16000**	**16000**	**16000**
glycerol	1663.7	1662.3	1654.4	1583.2	1334.4	1334.4
unreacted oil	866.7	1130.8	274.7(counted into product)	117.3	104.7	104.7
salts/waste from catalyst	218.9	13.3	13.3	4.0	4	20
water waste	7600				168.5	1035.8
total waste (catalyst+water)	7818.9	13.3	13.3	4.0	172.5	1055.8

6.2.1.1 Raw material input

According to Table 6.3, owing to the lower overall biodiesel yields Process 1, 2 require more feedstock but less alcohol to produce the same quantity of product than Process 4, 5, 6. This

has to be attributed to the different purification processes involved in these two clusters of processes. The conventional purification process in Process 1 and 2 removes the unreacted oil from biodiesel while the enzymatic polishing process in Process 4-6 converts the unreacted glycerides and FFA to biodiesel which consumes more alcohol but improves the overall conversion of oil. Because more feedstock can be saved than excessive alcohol consumed as shown in Table 6.3 and the cost of feedstock is higher than alcohol, Process 4-6 involving enzymatic polishing are more advantageous to the economy of biodiesel processes.

Process 3 needs no purification process because the product is already 'in-spec' after transesterification, which means the small amount of unreacted oil can exist with FAME as 'in-spec' biodiesel product to be sold and used as transportation fuel. Therefore, the mole conversion of oil can be regarded as 100 % in Process 3. As a result, Process 3 requires much less feedstock than Process 1 and 2. However, feedstock input in Process 3 is still higher than Process 4, 5 and 6 even though Process 3 has a higher mole conversion of oil. It can be explained by the larger molecular weight of FAEE-biodiesel from Process 4, 5 and 6 than FAME-biodiesel, which gains more credits in saving feedstock. Correspondingly the smaller molecular weight of methanol can benefit the demand for methanol in mass, which can be indicated by a much less alcohol input in Process 3 than those in Process 4-6, even though their mole conversions of oil are not that distinct.

6.2.1.2 Waste production

Among all processes, waste is mainly associated with the need for catalyst and water. Process 1 requires the most quantity of water because the alkaline catalyst can only be used for one single batch. The resulting waste is the salts from neutralization of sodium hydroxide used in the studied case which is cheap and most often used (Zhang et al., 2003). A large amount of water waste is also related to the salts as water is used for washing FAME, 47.5 kg/100 kg biodiesel (Sotoft et al., 2010). All of these contribute to the most total waste generated in Process 1. The water consumption/waste can be reduced and the salts can be sold as fertilizer if potassium hydroxide is used as catalyst instead, because the potassium sulphate or phosphate when neutralizing with sulphuric or phosphoric acid can be precipitated and the water can be more easily recycled (Sotoft et al., 2010).

For immobilized lipase-catalyzed processes (2, 3, 4, 5), the waste is mostly from the catalyst. The reuse capabilities of immobilized lipases allow much smaller quantities of them to be

required for the task. The productivity of the immobilized lipase determines the amount of catalyst needed as well as the amount of catalyst waste. Besides catalyst waste, Process 5 also has a small amount of water waste coming from the waste oil as feedstock.

Liquid lipase in Process 6 also requires reusability but less than immobilized lipases. So it has less catalyst waste than Process 2-5. Water is additionally required in 6 for lipase activation in Process 6 (Pedersen, 2011). As a result, Process 6 produces much more waste water than immobilized lipase-catalyzed Process 2-5. Additionally, due to the lower conversion of TAG (90 %) and higher FFA content (6.7 %) after transesterficiation by liquid lipase, the enzymatic polishing stage of Process 6 also contributes more water to the total waste than the same stage in Process 4 and 5.

As water is potentially recyclable and it is usually not considered when calculating E-factor (Sheldon, 2007), enzymatic processes still have an advantage over the chemical process of producing less waste.

6.2.2 Energy evaluation

As Figure 6.6 indicates, Process 1 requires the most energy among all processes. It is mainly related to the conventional purification process. Heavy duties are required on FAME distillation and removing water from glycerol by distillation.

For the rest of processes using heterogeneous catalyst, the step of water washing FAME is avoided which saves a lot of energy spent on glycerol distillation to remove water. That explains why the other processes have much lower energy consumption than Process 1.

The second highest energy consumption occurs to Process 2 owing to the heavy duty on FAME distillation. Because of the low efficiency of immobilized lipase working with methanol, Process 2 has the slowest reaction (48 hours) which results in the highest reaction energy for agitation.

Process 3 outstands itself with the least energy input among all processes mainly due to the eliminated purification process.

Because of the excess ethanol used in the enzymatic polishing stage, Process 4, 5 and 6 have higher energy burden on alcohol recovery than the other processes. However, it is still energy economical to adopt the enzymatic polishing stage rather than the conventional purification

process because the total energy spent on alcohol recovery and FAME distillation in Process 1, 2 are still higher than the energy spent on alcohol recovery in Process 4, 5 and 6.

Using waste oil as the feedstock in Process 5 requires a little more energy on removing the water from glycerol than Process 4. Process 6 requires more energy than Process 4 and 5 mainly because of the longer reaction time and the necessity of removing the added water for activating liquid lipase via glycerol distillation.

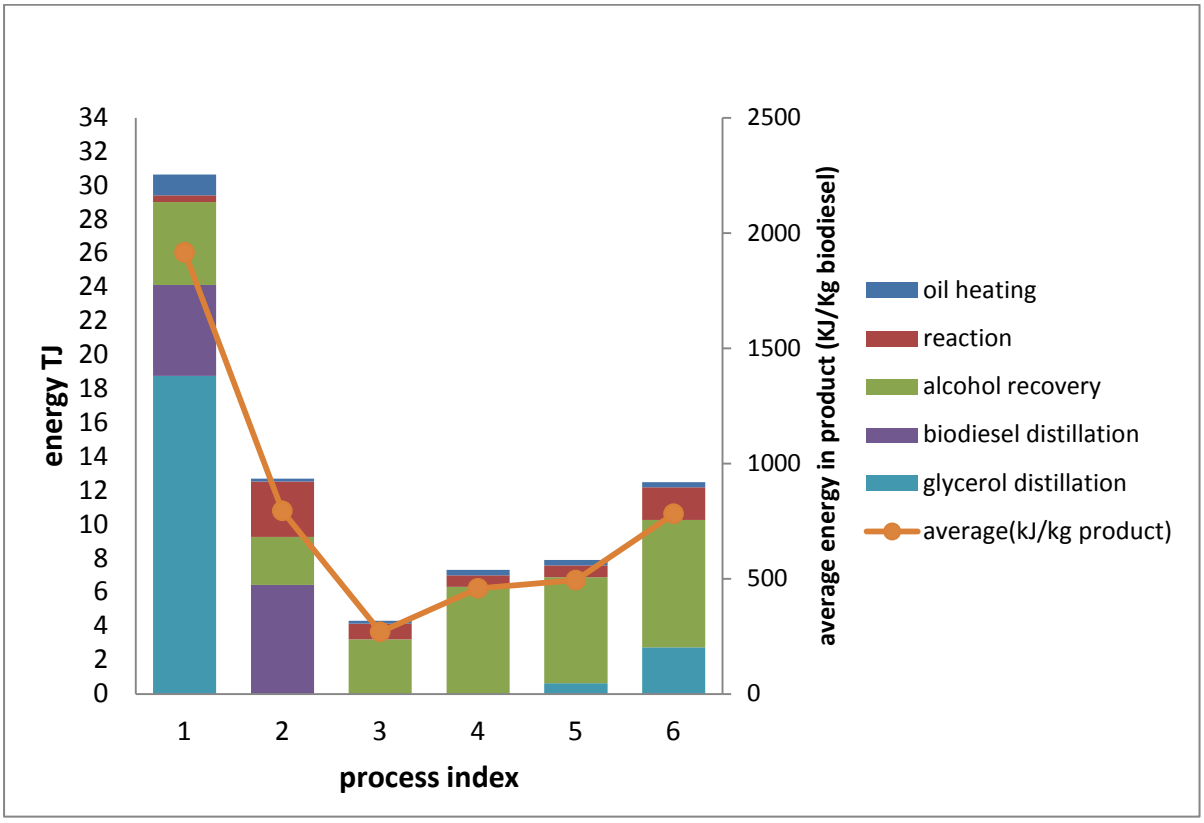

Figure 6.6 Energy consumption of each process

6.3 Conclusions and discussions

6.3.1 The important conclusions of process evaluations

First, the quantity of feedstock required in a process is dependent on the conversion rate of oil (biodiesel yield). The integration of enzymatic polishing stage can reduce the demand for feedstock by improving the yield of product, which is helpful to the economy of biodiesel processes.

Second, enzymatic processes produce much less waste due to the reusability of biocatalyst and no need of water for washing biodiesel product.

Third, the total energy consumption can be reduced by lowering water consumption in the process.

Fourth, having the enzymatic polishing stage can reduce the total energy consumption of the whole process, compared to the conventional purification process characterized by FAME distillation.

As a summary, the fully enzymatic processes (enzymatic transesterification + enzymatic polishing) are superior to the conventional chemical process with less feedstock input, waste production and energy consumption. The best process is the enzymatic process of in-spec product with no need for purification from the mass and energy consumption points of view.

However, it is worthwhile to mention that the fundamental basis of the whole evaluations of mass and energy are the economic equivalence of catalyst costs in the enzymatic and chemical processes, based on which is given the productivity of each catalyst. This backward induction method sets up the targets for enzymatic processes to be economically competitive with chemical process. Therefore, the productivity assumptions for immobilized lipases and liquid lipase are optimistic in a lack of sufficient research data support. The deviation of the catalyst productivity will change the catalyst input in the mass balance table and also change a great deal of the economic profile, which is critical to the economical viability of enzymatic processes. Discussions are given in the following paragraphs about the factors that can affect the catalyst productivity as well as other factors that can change the results of above evaluations.

6.3.2 Discussions about factors affecting evaluation results

The productivities of lipases are assumed to be irrelevant to the choice of reactor and operation, represented by batch STR in this evaluation. As a matter of fact, the operational stability, mechanical stability of the biocatalysts can be affected by the reactor and operations, which are discussed in details in the next chapter.

The quality of waste oil varies greatly in terms of contents of water, FFA as well as the quantity and kind of other contaminants, such as polymers, aldehydes and epoxides. The effects of waste oil on lipases will also vary accordingly. It is arbitrary to assume the same efficiency of lipase working on waste oil as refined oils. This uncertainty can affect the

productivity of the lipase and consequently affects the catalyst input and economy of the process, as discussed above.

Due to the soluble nature of liquid lipase, reuse of it is actually very difficult and it can possibly contaminate the biodiesel product although the limit for protein has not been included in any biodiesel specification. Since the liquid lipase is in the same phase of glycerol, reusing the mixture can realize the reuse of liquid lipase since glycerol does not pose any negative effect on the lipase. However, to some point the separation of lipase from glycerol is still necessary during the lifetime of liquid lipase due to the limitation of reactor volume as well as the need for recovering glycerol. Integration of membrane can possibly aid the separation of lipase from product but none of such studies has been reported so far. So it is not clear that how the membrane technology changes the energy profile of the process as well as the economic profile.

In practice, enzyme leaching from the carriers of immobilized lipases can also raise the risk of contaminating the product, which requires more energy on removing the protein possibly by heating.

CHAPTER 7

Discussion

7.1 Application of the methodology in other lipase-catalyzed biodiesel reactions

As introduced in section 3.3, chemical biodiesel process is normally composed of several process modules as listed in Table 7.1 and they are traditionally treated in chemical means. However, the drawbacks of these conventional methods can reduce the overall process efficiency. Lipases offer the alternative means to these process modules, which can improve the sustainability and efficiency of the processes. The proposed methodology has been validated with two of process modules transesterification and product purification (esterification) catalyzed by immobilized lipases. More interestingly, the methodology can be possibly applied on the other process modules to guide the process development.

Table 7.1 Conventional means for process modules involved in biodiesel productions (Dijkstra, 2010; Zhang et al., 2003; Brask et al., 2011)

Process modules	Means	Drawbacks
Oil degumming	Water degumming, acid degumming	Less efficient, corrosive (need high-quality steel in the construction materials)
Pretreatment	Distillation of FFA, acid catalysis	Energy-intensive, corrosive (need high-quality steel in the construction materials)
Transesterification	Alkaline catalysis	Saponification (need high-quality feedstock), low purity of by-product and heavy load of waste water
Product purification	Water washing and distillation of FAME	Energy-intensive, loss of unreacted oil

7.1.1 Enzymatic degumming

The phospholipids in the crude vegetable oils are divided into two classes, hydratable and non-hydratable. Water degumming, one of the traditional degumming methods, can deal only with hydratable phospholipids, whereas acid degumming, another commonly-used method in industry, can turn non-hydratable phospholipids to be hydratable (Dijkstra, 2010). As an alternative to acid degumming, enzymatic degumming has been gaining intensive interest since 1990s because this novel approach provides advantages, such as increased oil yield as the released products from phospholipids in the bulk of oil, reduction of wastewater by

decreasing the consumption of acid and alkaline, reduced energy and operation cost due to the mild reaction conditions and environmentally friendly nature (Jiang, et al., 2011).

Lipases, more specifically phospholipases, are the only kind of enzyme which has been commercialized for vegetable oil degumming, such as Lecitase®10L (pancreatic phospholipase A_2), Lecitase®Novo(phospholipase A_1 from *Fusarium oxysporum*), Lecitase®Ultra (phospholipase A_1 from *F.oxysporum* and *Thermomyces lanuginose*), Verenium's Purifine® (phospholipase C and Danisco's LysoMax® (lipid acyl transferase) (Jiang, et al., 2011).

It is necessary to modify the methodology for guiding the enzymatic degumming because the biocatalysts are in liquid formulations showing different requirements for the reaction conditions compared to immobilized lipase-catalyzed transesterification, e.g. emulsification for a larger reaction surface area and an optimal pH maintained by a buffer (Yang et al., 2006). These reaction characteristics put requirements on the reactor selection. Well-mixed reactors might be the choices, e.g. STR. High shear impeller should be used in a STR to create a stable emulsion of crude oil, buffer and enzyme solution.

7.1.2 Enzymatic pretreatment

Many interesting published studies have shown lipases are capable in lowering FFA content of low-quality but cheap feedstocks, for instance, waste cooking oil, acid oil and fatty acid distillate (side products from oil refinery) via esterification reaction and turning them to be suitable for alkali biodiesel production (Brask et al., 2011; Watanabe et al., 2007b). The pre-treatment can be done either with the whole acidic feedstock or separately with isolated FFA stream and then mix the treated stream with the oil stream (Brask et al., 2011).

This former process option shares the same reaction schemes as the second case, which can also be catalyzed by N435 due to its substrate specificity. This process however uses methanol due to the following conventional transesterification process using methanol and the inhibitory effect of methanol needs more caution in the process development because methanol has a lower solubility in this substrate mixture mainly composed of TAG and some FFA. On the contrary, the latter process can avoid such a problem because the solubility of methanol is much higher in the FFA stream (Du et al., 2007).

The reactor choices of these processes can be similar to both case studies. CSTRs and PBR can be sufficient to carry out the continuous operations in conjunction with the following continuous immobilized lipase-catalyzed transesterification.

Another alternative pretreatment is adding glycerol, the by-product from transesterification, into the acidic feedstock and converting FFA to monoglycerides and diglycerides, which will be later converted to biodiesel in the transesterification stage. This idea has been tested with chemical catalyst such as zinc chloride (Van Gerpen, 2004) and also with liquid *C. antartica* B lipase (Holm and Cowan, 2008). Due to the low reaction rate, the reactions have performed at high temperatures, 200 ^{o}C for chemical reaction and 65 ^{o}C for enzymatic reaction, and applying vacuum to release produced water. The advantage of this process lies in the utilization of by-product glycerol to increase the biodiesel yield.

7.2 Operational diagrams for each enzymatic process module

For a given feedstock with known FFA content, it is possible to use the diagrams below to guide the whole process to make 'in-spec' biodiesel. Most important operating conditions (e.g. alcohol addition, reaction time and enzyme loading) can be obtained from the operational diagrams by giving values to some variables.

Pretreatment of the high-acid feedstock is a necessary step before the alkaline transesterification. The conceptual diagrams in Figure 7.1 can be used to guide an easy operation of pretrement by knowing the initial FFA content and the target of residual FFA in the feedstock for alkaline transesterification.

First, the diagram in Figure 7.1 (a) indicates the correlation of the molar ratio of methanol relative to the initial FFA and the residual FFA target. Normally 5% FFA is the maximum residual content in the feedstock for alkaline transesterification. Subsequently, the molar ratio of methanol to initial FFA can be read from the diagram by identifying 5% in the X-axis.

Second, the diagram in Figure 7.1 (a) can be used to identify the optimal volumetric methanol addition to the oil by locating in the X-axis the initial FFA content, which is easy to measure or given by suppliers. When the initial FFA content is high, more methanol than the molar ratio of methanol to initial FFA guided by Figure 7.1 (b) needs to be added into the feedstock. It is because the esterification of high FFA content can cause more hydrolysis of TAG and generate DAG, which can compete with FFA to be reacted with methanol.

Third, for a given initial FFA content, the reaction time to achieve the target can be identified in the diagram in Figure 7.1 (c) with a known enzyme loading and vice versa.

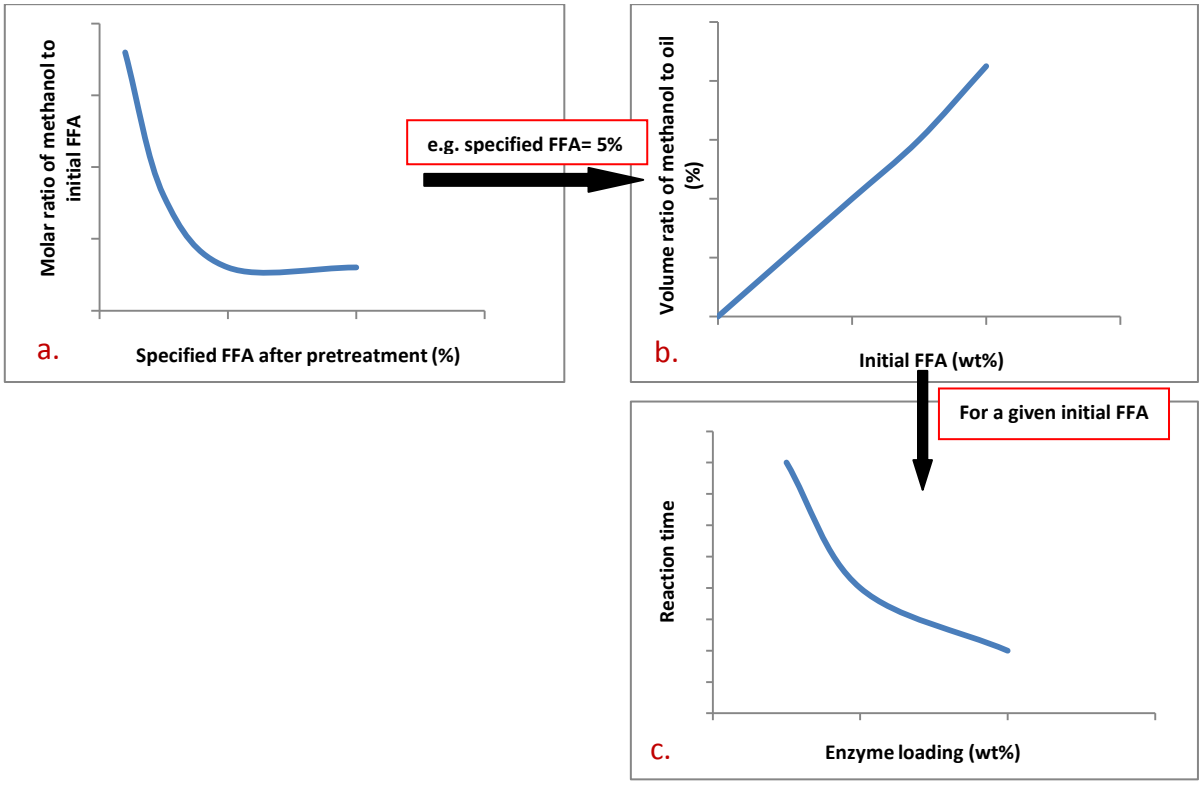

Figure 7.1 Diagrams for operating enzymatic pretreatment of feedstock

The enzymatic transesterfication is an alternative to alkaline transesterification. The product composition out of this reaction (e.g. FAEE and FFA contents) can affect the following purification process of product: the energy consumption on distillation of unreacted glycerides in conventional purification or alcohol consumption in enzymatic polishing process as discussed in Chapter 5.

The diagrams in Figure 7.2 indicate how to operate this reaction for preparing FAEE from refined vegetable oil and ethanol in a way imposing least consequence on the purification process. The diagram in Figure 7.2 (a) shows the equilibrium FAEE yields and the residual FFA contents after transesterification as function of the molar ratio of ethanol to oil. The equilibrium yield increases as the molar ratio of ethanol to oil and stabilizes at 95% since a molar ratio of 4.5. The corresponding residual FFA content stays at around 2%. Therefore, to achieve the optimal FAEE yield 95%, the diagram in Figure 7.2 (b) can guide the operation. In such a diagram, the reaction time can be identified by the given enzyme loading and vice versa.

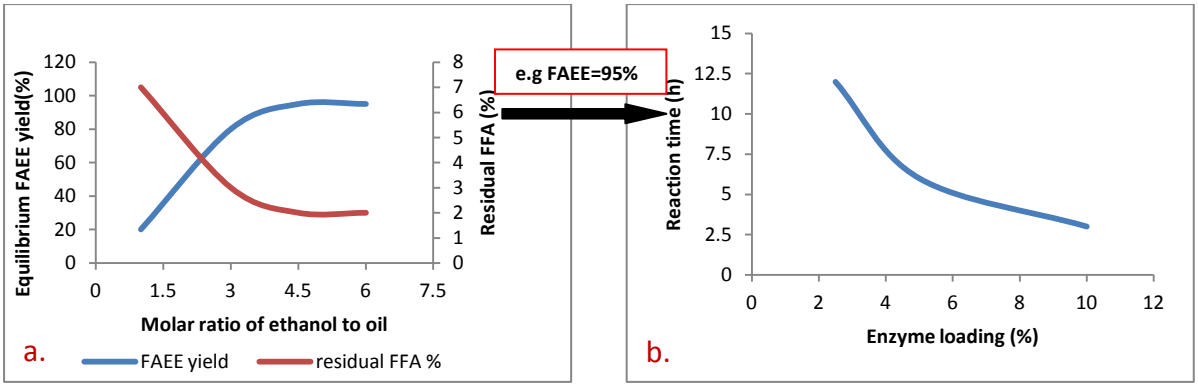

Figure 7.2 Diagrams for operating enzymatic transesterification

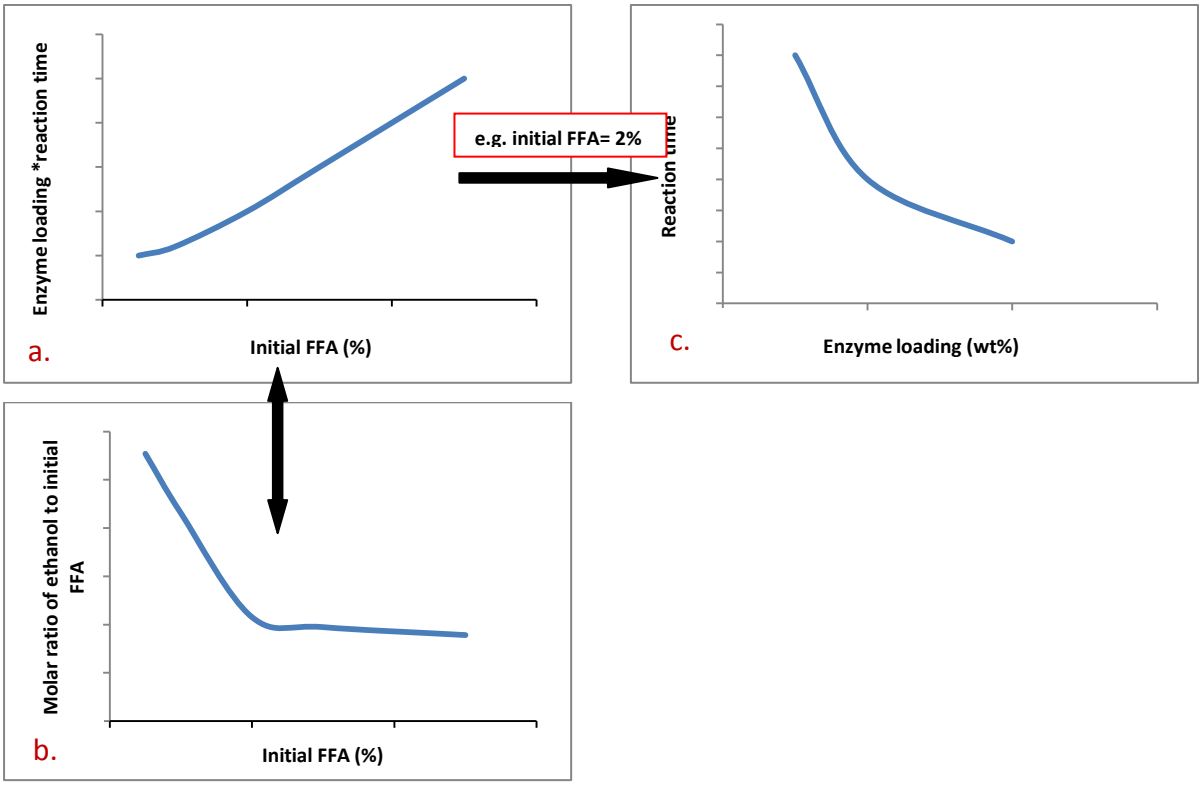

Figure 7.3 Diagrams for operating enzymatic polishing of biodiesel

The conceptual diagrams in Figure 7.3 can be subsequently used after the previous enzymatic transesterification. They are all based on the biodiesel specification for FFA content which should be below 0.25 wt% in the final product. Enzyme loading times reaction time and molar ratio of ethanol to oil can be read from diagrams in Figure 7.3 (a and b) after identifying the initial FFA content in the substrate for this reaction, which is residual FFA content in the product of previous transesterification. For an initial FFA content of 2%, the reaction time and enzyme loading can be looked up correlatly in the diagram in Figure 7.3 (c).

7.3 Guidelines for choosing appropriate reactors and scales

When applying the proposed methodology into real case studies, it is important to choose the right device of appropriate scale for experimental study at each step. They are not necessarily the miniature of the industrial reactors but they should be able to provide the best information. For the examples of immobilized lipase-catalyzed reactions, the fluid dynamics that can affect the mass transfer efficiency through multiple phases have been well presented in both lab and pilot scale reactors.

It is harmless to start with basic, most often-applied reactors for solid-liquid contacting reactions. Modifications can be made to the basic configurations after obtaining some understandings about the chemical and physical reaction characteristics as well as biocatalyst characteristics. The exact scales of them should be in the manageable and affordable domain; however, very often the choice is limited to the available reactors found by hand because new reactors are costly, especially the pilot-scale reactors.

Nevertheless, when a choice is affordable to make, the reactor and scale should be selected to serve the study purpose. For instance, when studying enzyme kinetics (e.g. activity and stability) and hydrodynamic characteristics (e.g. flow pattern, solid suspension), lab-scale STRs of volume about 100-250 mL are suitable and sufficient. The volume of a lab-scale PBR is not strict as long as a range of effective linear velocity (1-10 cm/min) can be achieved for evaluating the reactor efficiency and studying the mass transfer. Table 7.2 lists the common agitated devices and scales for studying biocatalysis with comments on their use.

Table 7.2 Comparison of common agitated devices and their scales for studying biocatalysis

Device	Scale	Advantages	Shortcomings
Vials in thermomixer	1.5 – 4 mL	High throughput, cheap	Poor mixing, impossible to study fluid dynamics
Shaking flask	20 – 100 mL	Medium throughput	Poor mixing; requiring expensive shaking equipment; impossible to study fluid dynamics
Lab STR	100 – 1000 mL	Sufficient to study fluid dynamics, intersolubility of reactants	Low throughput, difficult for process integration such as ISPR
Pilot STR	10-1000 L	Mimic the industrial conditions	Very low throughput, high material input, costly equipments

7.4 Generic plant for biodiesel production

Most of the existing biodiesel plants are committed to dealing with one specific kind of feedstock, which is often the most available refined vegetable oil. However, the availability of the feedstock can be limited to its seasonal cultivation. Therefore, a capability of utilizing variable feedstocks can protect the capacity of the plant, which can be realized by a generic plant.

The biodiesel production process varies corresponding to the different feedstock quality as well as the method applied in the transesterification. Most of the large scale biodiesel plants are based on alkali catalysis and they can be modified to generic plants for running a variety of feedstocks. One such example is shown in Figure 7.4 and an example of enzymatic generic plant is shown in Figure 7.5. Feedstocks of at least three different qualities (refined oil, crude oil and waste oil) can be processed in such generic plants by directing them through the required process modules. The realization and success of such generic plants require the support of adequate process control systems to ensure the process targets.

The realization of generic plants can improve the flexibility as well as the sustainability by being able to utilize waste oil. However, the production cost will be increased by adding a number of extra process modules. Therefore, an economic evaluation is probably needed to find out how generic the plant can possibly, i.e. how many kinds of feedstocks are economically realistic to be handled in such plants.

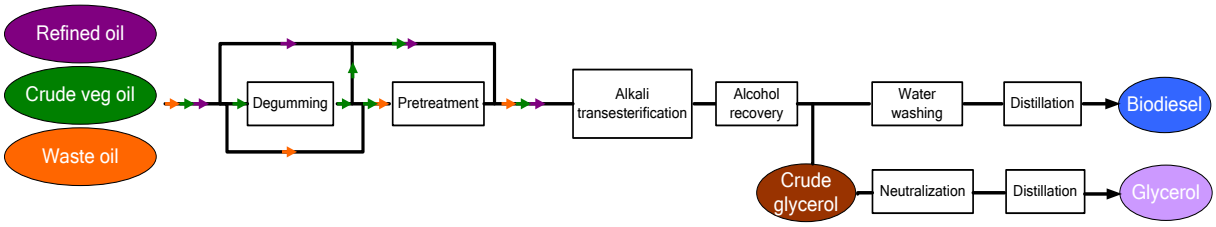

Figure 7.4 A generic plant for biodiesel production via alkaline catalysis

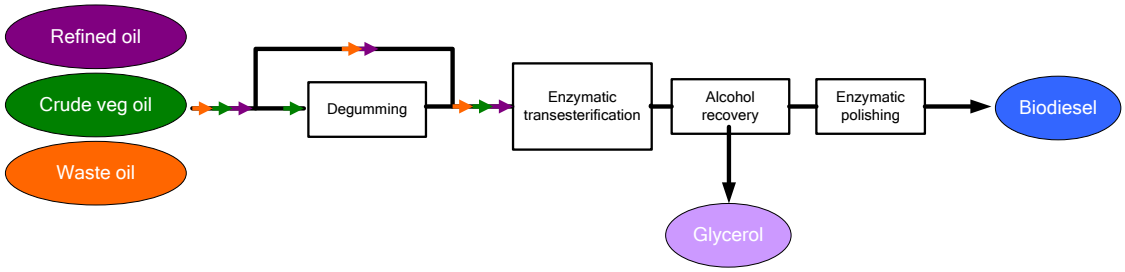

Figure 7.5 A generic plant for biodiesel production via enzymatic catalysis

7.5 Alternative reactors for immobilized lipase-catalyzed reactions

Besides STR and PBR, some other reactors have also been reported for the reactions catalyzed by immobilized lipases, such as fluidized bed and bubble column (Ricca et al., 2009; Hilterhaus et al., 2008). They share some common characteristics as STR or PBR, competent to provide adequate reaction environment determined by the characteristics of immobilized lipase-catalysis. In addition, this section also addresses some non-conventional reactors for carrying out this type of reactions, which show promises for large-scale application.

7.5.1 Fluidized bed reactor (FBR)

As another type of well-mixed reactor, FBR is a fluidizing heterogeneous catalyst bed by the rapid up flowing stream of the substrate or assisted by a gas or a second liquid stream (Trambouze and Euzen, 2002). For an efficient operation the particles should be of nearly uniform size, as Figure 7.6 shows. The vigorous mixing of the solid catalyst with the viscous oil can be achieved in FBR, leading to an excellent contact of the immobilized lipase and lipids. But the particles can experience less shear stress in this reactor than STR.

FBR has not been well studied for the immobilized lipase-catalyzed biodiesel reaction with only one reported work (Ricca et al., 2009). For the large scale application there are some problems need to be solved, such as low conversion, loss of catalyst in the product stream (Sotoft et al., 2010).

7.5.2 Bubble column reactor (BCR)

When the gas is sparged from the bottom of the column into either a liquid phase or a liquid–solid suspension, it is a bubble column reactor, named from bubble-like large pockets of gas,

free of particles, rising through the bed. The liquid and solid phases are considered to be well mixed in such a reactor and the gas phase moving as plug flow (Trambouze and Euzen, 2002).

This reactor is advantageous for the reactions involving highly viscous reactants. But the gas feature makes the reactor not suitable for reactions having volatile reactants, but is perfect for shifting the thermodynamic equilibrium of reaction by instantly removing product. A successful example of combining the two advantages is the production of surfactant in BCR from esterification of polyglycerol-3 and lauric acid catalyzed by N435, where the formed water is removed by pressurized air rising through the reactor (Hilterhaus et al., 2008).

7.5.3 Expanded bed reactor (EBR)

PBR has inherent problems of high pressure drop and bed clogging by contaminates in the substrates or viscous products. To overcome these problems and enhance the reactor efficiency, some modified reactors have been developed and applied in bioprocesses, i.e. expanded bed reactor. Although it has more complicated principles, it offers solutions to heterogeneous biocatalysis and it is promising for large scale applications.

The performance of EBR lies between PBR and FBR. It is like a stable fluidized bed (lower back mixing) loaded with variable particle sizes and densities, which aims to achieve a gradient of voidage, flow velocity and concentration through the bed. The larger particles populate the lower portion of the bed while the smaller particles populate the upper portions, as Figure 7.6 shows. As a result, EBR has a smaller chance of bed clogging by contaminates or viscous reactants. Therefore, EBR is promising to be used for converting waste oil to biodiesel by immobilized lipases without a pretreatment of waste oil. However, there has not been any reported work of immobilized lipase-catalysis in EBR.

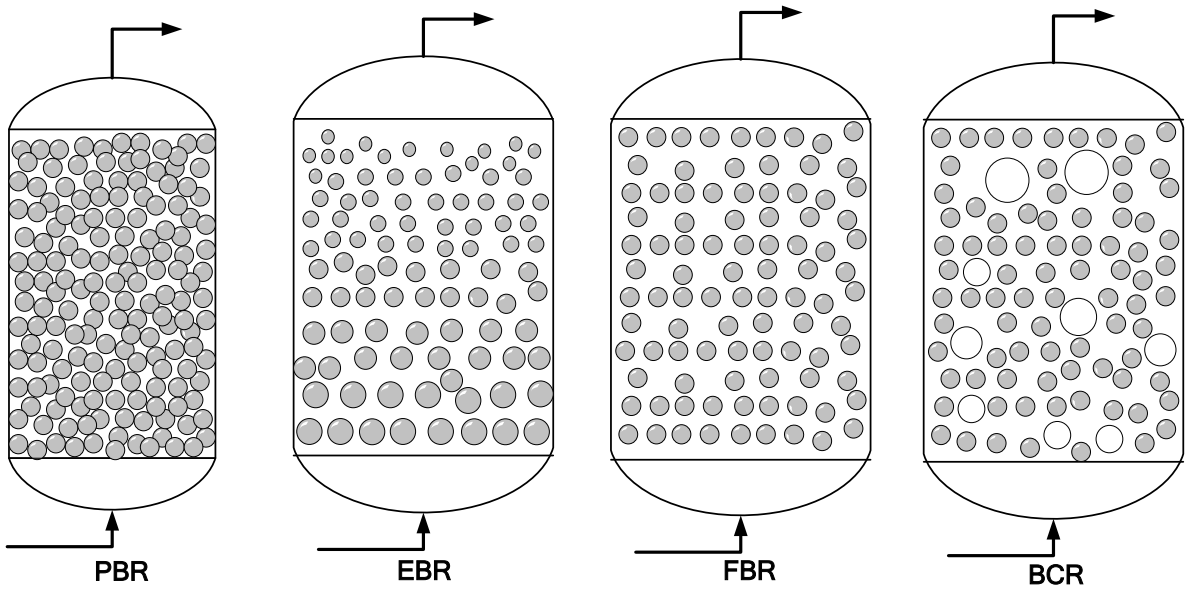

Figure 7.6 Schematic diagrams of column reactors

7.5.4 Comparison of reactors

Schematic diagram of column reactors (PBR, FBR, EBR and BCR) is shown in Figure 7.6. The comparison of reactors addressed in this thesis is given in Table 7.3. Knowing the differences of reactors is useful to the reactor selection for a specific reaction process catalyzed by immobilized enzymes. As the table show, STR, PBR and EBR have more prominent characteristics than FBR and BCR, which show some neutral behavior.

Table 7.3 Comparison of reactors (Levenspiel, 1999; Buchholz et al., 2005; Trambouze and Euzen, 2004)

Reactor type	Space-time-yield	Particle size	Mass transfer efficiency	Pressure drop	Damage to catalyst	Power input
STR	low	not specific, larger than filter hole	high	little	high	high
FBR	medium	uniform	high	medium	medium	medium
PBR	high	preferably uniform	low	high	low	medium
EBR	medium	gradient	low	low	low	low
BCR	low	uniform	medium	high	medium	medium

CHAPTER 8

Conclusions

8.1 Conclusions of case studies

Two immobilized lipase-catalyzed processes (transesterification and esterification) have been taken as examples for validating the proposed methodology. The central conclusions of the two case studies are presented here.

8.1.1 Case 1: immobilized lipase-catalyzed transesterification

8.1.1.1 Reaction scheme and characteristics

The overall reaction scheme for this case is TAG + 3EtOH ↔ 3FAEE + Glycerol, and 96% ethanol is the primary choice of alcohol for this reaction.

The intermittent reaction steps include: TAG + EtOH ↔ FAEE +DAG; DAG + EtOH ↔ FAEE + MAG; MAG + EtOH ↔ FAEE + Glycerol

Key reaction characteristics of this case have been obtained and presented as follows:

First, it is a multiple phases coexisting reaction system composed of solid particles of immobilized lipase, a lipid phase and additionally a polar phase when alcohol exceeds the solubility limit or when the by-product glycerol is present.

Second, ethanol has a solubility limitation in the oil substrate (about 0.5 eq in the refined rapeseed oil) and the un-dissolved ethanol can inhibit the lipase.

Third, the hydrophilic phase formed by glycerol can potentially cause the mass transfer limitation by clogging the carriers of the immobilized lipases.

8.1.1.2 Biocatalyst and reactor selection

The abovementioned reaction characteristics place requirements on the selection of immobilized lipase as well as the reactor selection, meaning the selected biocatalyst and reactor should be able to deal with these problems.

TLL has shown high activity and stability with 96% ethanol. The carrier for immobilizing TLL should be hydrophobic owing to its low affinity for glycerol, which results in the biocatalyst NS 88001.

95% FAEE can be obtained after a reaction time of 6 hours with 5 wt% NS 88001 loading, 1.5 eq ethanol at 35 ^{o}C and the same product yields can be obtained for at least 5 batches of repeated use.

As heterogeneous reactors, STR and PBR have been evaluated for this reaction. The stepwise addition has been adopted in batch STR and PBR to avoid the enzyme inhibition by excess un-dissolved ethanol.

STR is advantageous at improving mass transfer, while mechanical damage to immobilized lipases can be its weakness. Above 80% particles were found intact after being stirred for 120 hours at a P/V close to an industrial application (1.0 W/L).

PBR has less damage to immobilized lipases but the pressure drop limitation can cause insufficient mass transfer. A linear velocity of 7.6 cm/min in the lab-scale PBR has achieved an overall productivity of 2.52 kg FAEE(kg enzyme)$^{-1}$h^{-1}, close to the efficiency of a STR under similar conditions.

A combination of CSTR+PBR can couple the advantages of each other and achieve an efficient continuous process.

8.1.1.3 Validation in pilot plant

The batch transesterification in lab-scale STR has been successfully scaled up to a pilot-scale STR with a constant power input per volume. A similar catalyst performance was obtained in the pilot-scale STR in terms of initial rates, reaction progress. High stability of NS 88001 was observed with 96% ethanol through 4 repeated batches in the pilot-scale STR. Higher mechanical damage was found in pilot-scale STR with only 74% intact particles at the same conditions tested in lab. The size distribution of broken particles was found far from the possibility of clogging the filter for retaining the catalyst in the reactor.

The catalyst performance at a scaled STR can be accurately predicted by that in a smaller STR.

8.1.2 Case 2: immobilized lipase-catalyzed esterification

8.1.2.1 Reaction scheme and characteristics

The major reaction is FFA + EtOH ↔ FAEE + H_2O and the other reactions include DAG + EtOH↔ FAEE + MAG; MAG + EtOH↔ FAEE + Glycerol.

This reaction system shares some common characteristics as first case study. It is also a multi-phasic system but the reaction mixture is much less viscous. It also allows a much larger amount of ethanol to be dissolved, which can be used for pushing the reaction towards the product side.

Instead of glycerol, water is the major by-product and should be removed to drive the reaction to the right side. Furthermore, any external water should be avoided from the reaction system.

8.1.2.2 Biocatalyst and reactor selection

Novozym 435 (immobilized CalB lipase) is selected due to its high activity in anhydrous condition and its specificity for FFA and partial glycerides.

Mass transfer limitation is less a problem in this reaction system but the efficiency of water removal is more critical. The integration of water removal strategy into the reactor or the whole process requires some designing considerations. Both STR and PBR have succeeded in reducing FFA content to specified level via a few steps with water removal by vacuum drying as an intermittent step. 4-step PBR process can result in a biodiesel product with all major components within specifications.

8.1.2.3 Validation in pilot plant:

The pilot-scale reaction also managed to suppress the FFA content below 0.25% but the partial glyceride contents were still beyond the specified levels.

8.1.3 Process evaluations

The higher biodiesel yield is obtained in the process, the less feedstock input is needed. Consequently, the enzymatic polishing can reduce the feedstock quantity by improving the biodiesel yield.

Compared to conventional alkaline process, immobilized lipase catalyzed process has less waste water because of the eliminated need of removing alkaline catalyst by washing biodiesel. As another consequence of using less water, the energy consumptions for enzymatic processes are also much reduced. Energy consumption is further reduced with the advantage of no need of distilling biodiesel in enzymatic processes.

Therefore, among the evaluated processes, the process composed of an enzymatic transesterification followed by an enzymatic esterification saves most feedstock while the process of enzymatic transesterification with 'in-spec' product and no need for purification is most energy-economic.

8.2 Conclusions of methodology

A methodology framework has been proposed for evaluating and implementing a biocatalytic process. It has been validated by two immobilized lipase-catalyzed reactions in this work. The proposed methodology provides a clear guidance for the process development and aids the process evaluation in a systematic way.

The most important elements of a process can be identified by going through those steps and the suggested tools of each step can effectively direct the experimental work in a small scale to concrete each element. The methodology also emphasizes the need of validating the developed process in a pilot plant, which can verify the possibility of predicting the process efficiency of a larger scale. Besides the technical challenges being addressed, the methodology also involves process evaluation from mass and energy perspectives, which can help drawing an economic profile and assessing environmental risks of the process.

Although this methodology has only been applied to the immobilized lipase-catalyzed reactions, it is possible to apply this methodology to the evaluation and implementation of other biocatalytic reactions which have the similar characteristics as the studied cases. The methodology developed in this work also shows an example of developing the next generation of process technology.

CHAPTER 9

Future work

If there are more sources (time, funding etc.), some more tasks can be carried out to improve and expand the topic of this work. They are suggested in this chapter.

9.1 Modification to the studied reaction systems

One of the challenges of the biodiesel case studies is the productivity of the immobilized lipases. An important factor influencing the productivity is the physical life time of the immobilized lipases, which is related to the carriers. Therefore, solutions should be provided to reduce particle damages and enable sufficient reuse of the immobilized enzyme. To that end, the following suggestions can be useful.

9.1.1 Modification to agitation means

Low-shear impellers that can serve the purposes of solid suspension and liquid blending should be tested in a STR, e.g. hydrofoil (Paul et al., 2003).

Sonication has been recently applied in enzymatic transesterification in a small scale (25 mL) (Kumar et al., 2011) and it was found that the ultrasonic cavitational mixing was effective of a low power input (0.1 W/L) and greatly reduced the reaction time to 30 min. It is interesting to optimize this process in the light of biodiesel yield and test this effect in a larger scale.

9.1.2 Expanded choice of reactors

As well-mixed reactor and plug flow reactor respectively, FBR and EBR have low shear stress to the immobilized enzymes. Therefore, they can possibly be alternatives to STR and PBR for carrying out the enzymatic biodiesel reactions. Novel reactors can be specially designed for immobilized lipase-catalyzed reactions with solutions to both the particle damage and mass transfer problem through multiple phases.

9.1.3 Development of new carriers for immobilization

The development of carrier should focus on the improvement of rigidness to resist higher pressure drop and shear stress. Furthermore, an increased capacity for enzyme/protein loading should also be an important consideration of carrier development.

9.2 Develop tools to aid the implementation of methodology

As useful tools, modeling and simulations can save the cost and time on carrying out experiments allowing more efficient evaluations of biocatalyst or reactor options (Tufvesson et al., 2011). However, the tools have not been widely applied in biocatalytical processes probably because the research on thermodynamics of biological systems is far behind the need for itself, which makes process modeling and simulation extremely difficult.

9.2.1 Kinetic modeling for biocatalyst evaluation

Kinetic models of immobilized lipases can facilitate the evaluation of biocatalysts with different reaction conditions. Many models have been reported but most of them have the difficulties of accurately predicting the biocatalyst performance with some non-stoichiometric alcohol loadings, e.g. 2.0 eq and 0.3 eq (Xue, 2008). A better understanding of the effect of alcohol is needed to improve the models. For that purpose, establishing an analytical method for tracing the alcohol concentration in the reaction system can be helpful to investigating the mechanism.

9.2.2 Hydrodynamic modeling for reactor evaluation

Adequate hydrodynamic models can interpret the interactions of hydrodynamic conditions provided by reactors and biocatalyst performance. They can accelerate the reactor evaluations and also define the operation limits for a given biocatalyst.

It is useful to establish a mass transfer model within PBR because it can guide the use of flow velocity in the column to provide a sufficient mass transfer to the reaction within pressure drop limitation.

9.3 Develop methods for using tools

Tools are suggested alongside the methodology to provide relevant information to each step. However, the methods for how to use these tools are not sufficiently given. Thusly, efforts can be put on developing methods in the future to enforce the effectiveness of tools and aid the application of the methodology.

They can be used for the lab-scale investigation, such as how to perform the phase behavior test. The developed dyeing method for visualizing the glycerol can only qualitatively assess the effect of glycerol on immobilized lipases. It would be more useful for screening carriers if any tool is available for quantitatively measuring the effect.

They can also be for a larger-scale study. In the pilot-scale STR, it is not so easy to obtain the just suspended speed for the immobilized lipase by visual observation. Therefore, methods utilizing reflection of ultrasound signal from particles, or response of solid concentration variance at a specific position inside the tank can be applied when the visual observation is not possible (Paul et al., 2003).

9.4 Process control to realize a generic plant for the flexibility of using different feedstocks

As introduced in 7.2, a generic plant is aimed to entitle the process better flexibility and higher capacity for handling feedstocks of different qualities or different kinds.

The realization of this purpose needs the implementation of a sufficient process control system to deal with the changes to the process introduced by feedstocks and ensure the consistent product quality.

The establishment of process control systems demands the identification of input variables and measurable responses. FFA level in the feedstock and ethanol dosing are most interesting input variables, which can affect the process layout and biocatalyst productivity. Challenges lie in finding out which responses to analyze and whether direct or indirect response to use. Answers to these questions depend very much on the availability of analytical methods, preferably on-line measurements, i.e. on-line HPLC or NIR spectroscopy.

9.5 Operational windows

A conceptual operational window for PBR is given in the example of enzymatic biodiesel process in this thesis. Key process parameters are qualitatively presented in such windows guiding the process design and optimization. However, the quantitative boundary-defined windows are more useful to scaling up a specific process. To prepare such quantitative windows for enzymatic biodiesel process, more work will be needed, experimental work and reactor modeling.

Although the operational window for STR is quantitatively presented in Paper 2, the relation of the stirring effect and the glycerol inhibition has not been quantified. It can be investigated by altering the stirring speed in a reaction mixture containing a stoichiometric amount of glycerol.

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Appendix A

High-Performance Liquid Chromatography (HPLC) analytic method

A1.1 Sample preparation and HPLC method description

Samples (50 μL) were dissolved and diluted in a mixture of 0.5 mL acetic acid and n-heptane (4:1000, v/v) and further diluted 100-fold in the same mixture to achieve concentrations of around 1.0 mg mL^{-1}. 40 μL of the solution was injected on an HPLC (Dionex A/S, Hvidovre, Denmark) for analysis of the composition of FAEE, TAG, FFA, DAG and MAG. The HPLC was equipped with U3000 autosampler, TCC-3000SD column oven and U3400A quaternary pump modules. A Corona® Charged Aerosol Detector (Thermo Scientific Dionex, Chelmsford, MA, USA) was used for detection with nitrogen at an operating pressure 35.0 psi. The separation was done on a 250 mm × 4.0 mm cyanopropyl column (Discovery® Cyano from Sigma–Aldrich, Steinheim, Germany) at a flowrate of 0.75 mL min^{-1}. Program control, data acquisition, and analysis were carried out using Chromeleon 6.8 software. A binary gradient program was applied using phase A: 99.6% n-heptane, 0.4% acetic acid; and phase B: 99.6% t-butyl methyl ether, 0.4% acetic acid (Foglia, 1997).

An HLPC-CAD chromatogram example is shown in Figure A1 below.

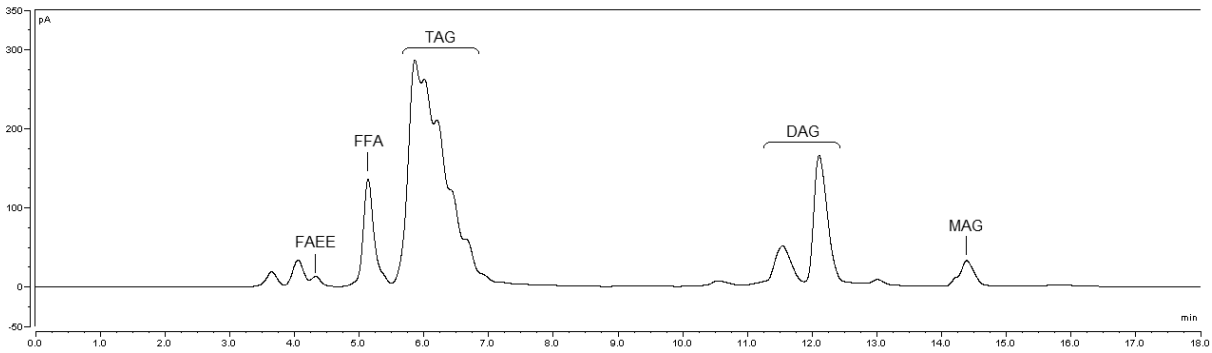
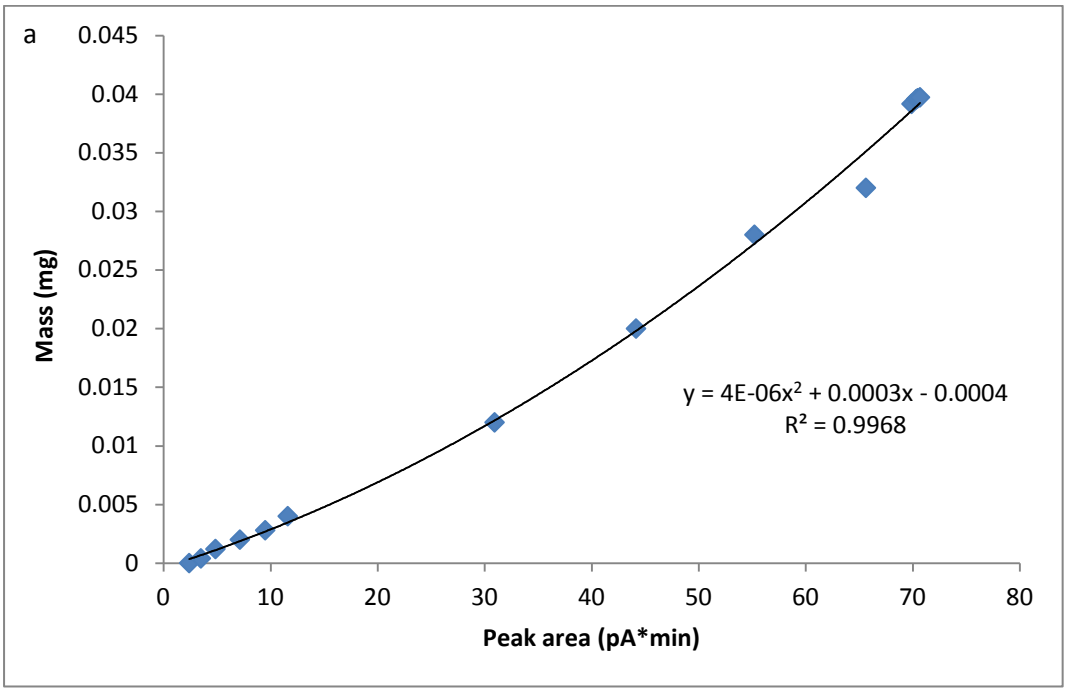
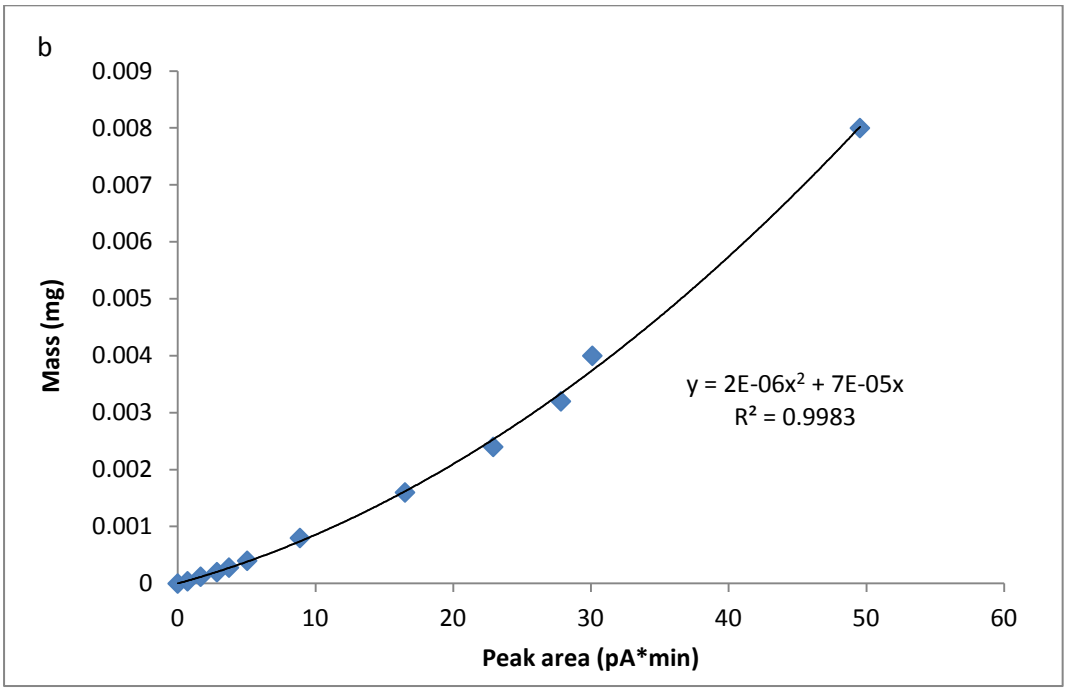


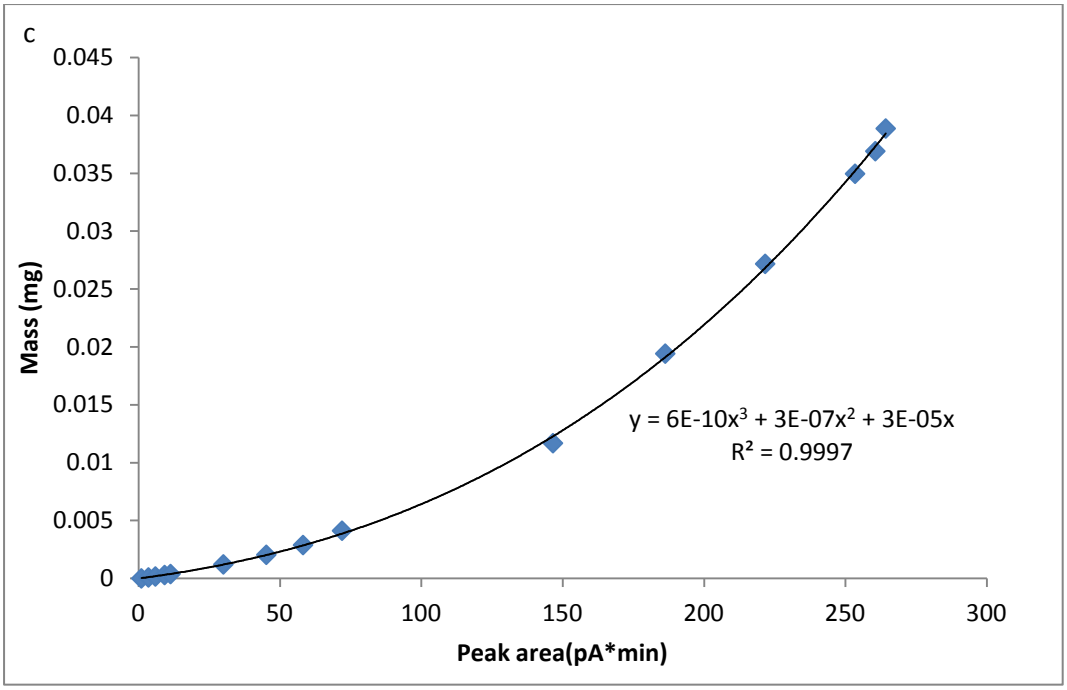
Figure A1 HPLC-CAD chromatogram of a biodiesel sample.

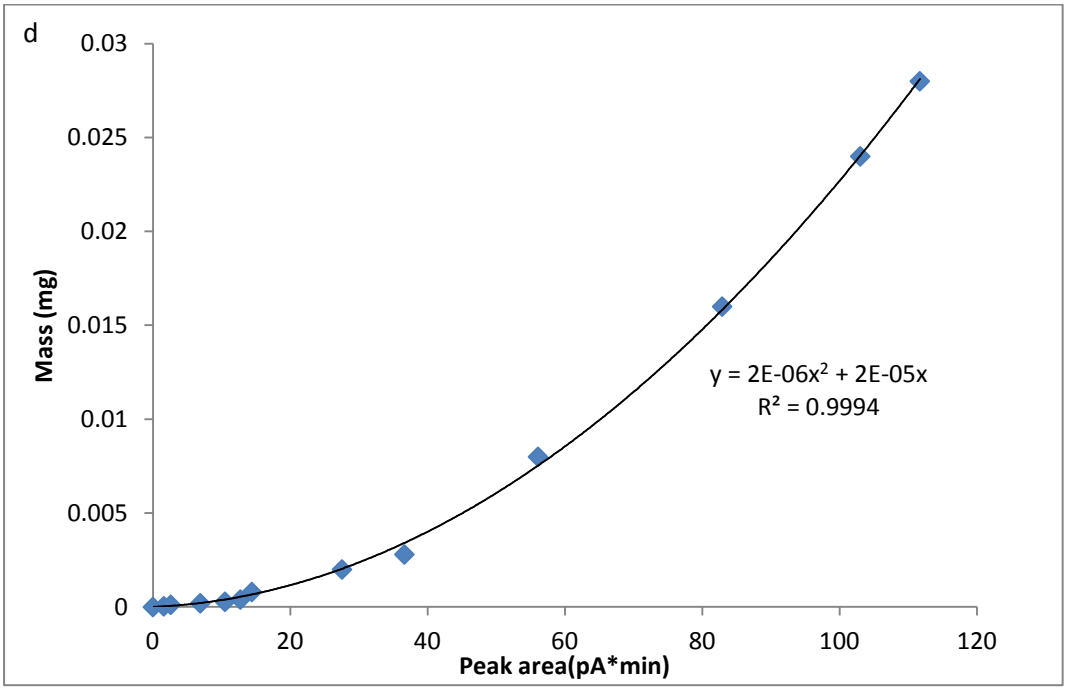
A1.2 Calibration of major components

The calibration curves of five major components in the biodiesel samples are presented in Figure A2. The mass of each component in the injected solution can be determined by putting the the CAD response in the form of peak area into the corresponding calibration curve.






c
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Mass (mg)
y = 6E-10x³ + 3E-07x² + 3E-05x
R² = 0.9997
0
50
100
150
200
250
300
Peak area(pA*min)


d
0.03
0.025
0.02
0.015
0.01
0.005
0
Mass (mg)
y = 2E-06x² + 2E-05x
R² = 0.9994
0
20
40
60
80
100
120
Peak area(pA*min)

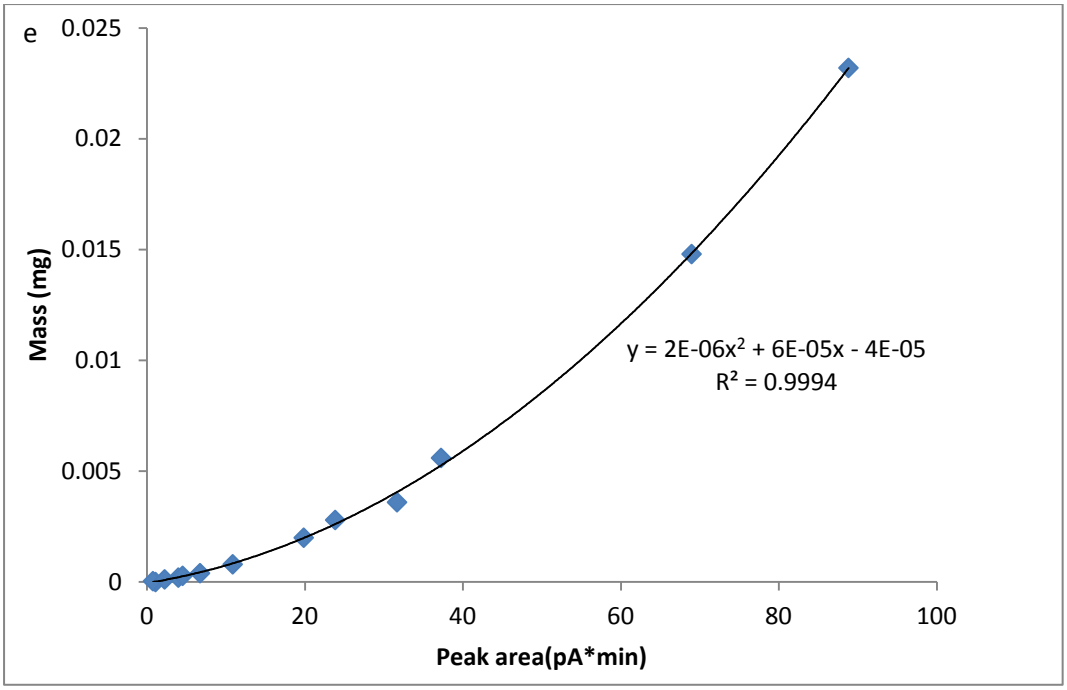


Figure A2 Calibration curves for a. ethyl oleate (FAEE), b. oleic acid (FFA), c. rapeseed TAG, d. 1,3-diolein (DAG), e. 1-monooleoyl-rac-glycerol (MAG)

A1.3 Calculation of FAEE yield

Yield of biodiesel is defined as FAEE mass percentage in the mixture of FAEE and glycerides including TAG, DAG, MAG and FFA as indicated by the equation below.

$$\text{Yield(mass\%)} = \frac{\text{FAEE}}{\text{FAEE+TAG+DAG+MAG+FFA}} * 100\%$$

Appendix B

Biodiesel standard----EN 14214

EN 14214 is a European Standard that describes the requirements and test methods for FAME, the most common type of biodiesel. Biodiesel fuels can also be other alkyl esters, such as FAEE produced from ethanol. However, these types of biodiesel are not covered by EN 14214 which applies only to methyl esters i.e. biodiesel produced using methanol.

The current version of the standard was published in November 2008 and supersedes EN 14214:2003.

Table 1 — Generally applicable requirements and test methods

Property	Unit	Limits		Test method [a] (See Clause 2)
		minimum	maximum	
FAME content [a]	% (*m/m*)	96,5 [b]	–	EN 14103
Density at 15 °C [c]	kg/m^3	860	900	EN ISO 3675 EN ISO 12185
Viscosity at 40 °C [d]	mm^2/s	3,50	5,00	EN ISO 3104
Flash point	°C	101	–	EN ISO 2719 [e] EN ISO 3679 [f]
Sulfur content	mg/kg	–	10,0	EN ISO 20846 EN ISO 20884
Carbon residue (on 10 % distillation residue) [g]	% (*m/m*)	–	0,30	EN ISO 10370
Cetane number [h]	–	51,0	–	EN ISO 5165
Sulfated ash content	% (*m/m*)	–	0,02	ISO 3987
Water content	mg/kg	–	500	EN ISO 12937
Total contamination	mg/kg	–	24	EN 12662
Copper strip corrosion (3 h at 50 °C)	rating	class 1		EN ISO 2160
Oxidation stability, 110 °C	hours	6,0	–	prEN 15751 [i] EN 14112
Acid value	mg KOH/g	–	0,50	EN 14104
Iodine value	g iodine/100 g	–	120	EN 14111
Linolenic acid methyl ester	% (*m/m*)	–	12,0	EN 14103
Polyunsaturated (≥ 4 double bonds) methyl esters	% (*m/m*)	–	1	[k]
Methanol content	% (*m/m*)	–	0,20	EN 14110
Monoglyceride content	% (*m/m*)	–	0,80	EN 14105
Diglyceride content	% (*m/m*)	–	0,20	EN 14105
Triglyceride content [a]	% (*m/m*)	–	0,20	EN 14105
Free glycerol	% (*m/m*)	–	0,02	EN 14105 [l] EN 14106
Total glycerol	% (*m/m*)	–	0,25	EN 14105
Group I metals (Na+K) Group II metals (Ca+Mg)	mg/kg mg/kg	–	5,0 5,0	EN 14108 [l] EN 14109 EN 14538 EN 14538
Phosphorus content	mg/kg	–	4,0	EN 14107

[a] See 5.6.1.

[b] The addition of non-FAME components other than additives is not allowed, see 5.2. When C17-methyl esters naturally appear in FAME this can result in a lower measured fatty acid methyl ester content. In this situation reference should be made for verification to a modified determination procedure [4], until a modified method is established within CEN.

[c] Density may be measured by EN ISO 3675 over a range of temperatures from 20 °C to 60 °C. Temperature correction shall be made according to the formula given in Annex C. See also 5.6.2.

[d] If CFPP is -20 °C or lower, the viscosity shall be measured at -20 °C. The measured value shall not exceed 48 mm^2/s. In this case, EN ISO 3104 is applicable without the precision data owing to non-Newtonian behaviour in a two-phase system.

[e] Procedure A to be applied. Only a flash point test apparatus equipped with a suitable detection device (thermal or ionization detection) shall be used. See also 5.6.2.

[f] A 2 ml sample and apparatus equipped with a thermal detection device shall be used.

[g] ASTM D 1160 shall be used to obtain the 10 % distillation residue. See also 5.3.4.

[h] See 5.6.3.

[i] See 5.6.2.

[k] A suitable test method is under development by CEN [3].

[l] See 5.6.2. See Annex A for precision data for sum of Na + K.

Appendix C

A pilot plant for enzymatic biodiesel production

C1.1 PID of pilot plant

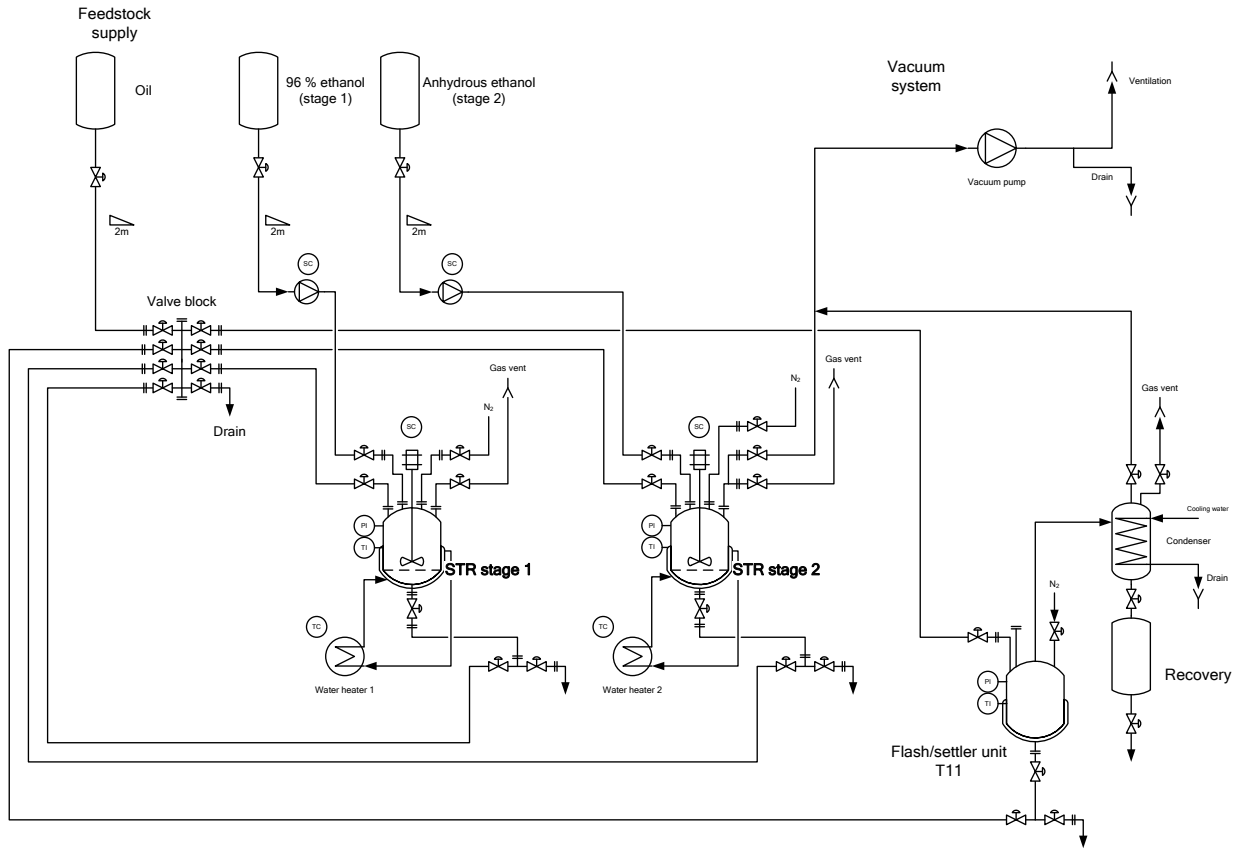


Figure C1 PID of pilot STR process

C1.2 Photos of pilot plant

Figure C2 Pilot-scale STR (20 L)

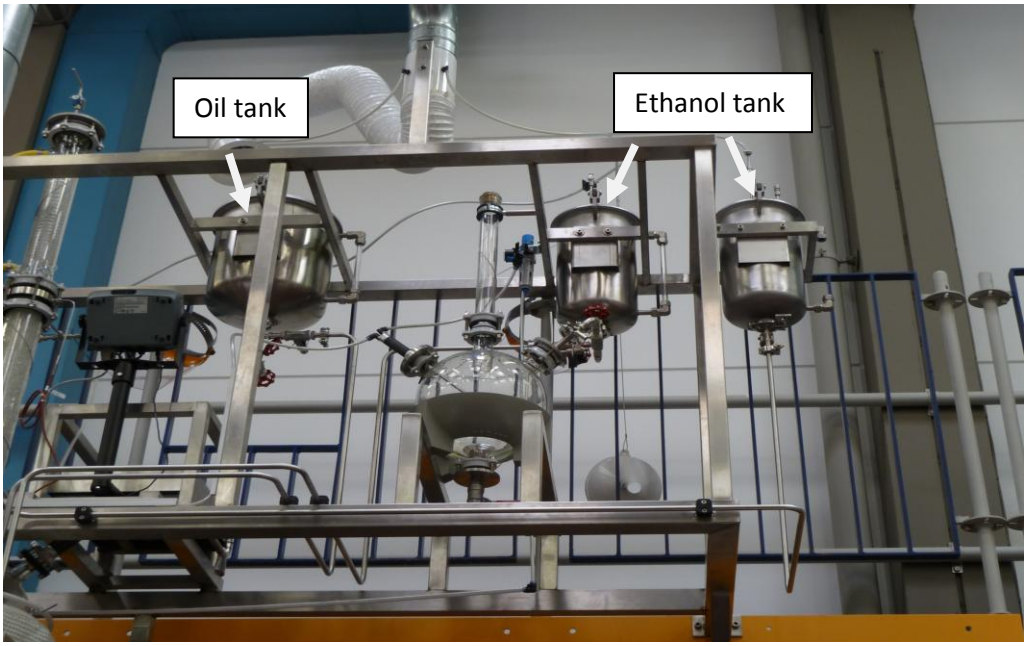


Figure C3 Substrate tanks

Appendix D

Publications

PAPER 1

In situ visualization and effect of glycerol in lipase-catalyzed ethanolysis of rapeseed oil

Authors: Yuan Xu, Mathias Nordblad, Per M. Nielsen, Jesper Brask and John M. Woodley

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In situ visualization and effect of glycerol in lipase-catalyzed ethanolysis of rapeseed oil

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Dyeing

ABSTRACT

Immobilized lipases can be used in biodiesel production to overcome many disadvantages of the conventional base-catalyzed process. However, the glycerol by-product poses a potential problem for the biocatalytic process as it is known to inhibit immobilized lipases, most likely by clogging of the catalyst particles. In this paper, this negative effect was further investigated and confirmed in ethanolysis of rapeseed oil. A dyeing method was developed for *in situ* visualization of glycerol in order to study its partitioning and accumulation during the ethanolysis reaction. The method was used to illustrate the interaction of glycerol with immobilized lipases and thus provided an aid for screening supports for lipase immobilization according to their interaction with glycerol. Glycerol was found to have great affinity for silica, less for polystyrene and no affinity for supports made from polymethylmethacrylate and polypropylene. It was also found that the immobilization of enzyme on the support influenced the adsorption of glycerol to the surface of the enzyme carrier.

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1. Introduction

The depletion of fossil fuels makes it increasingly necessary to develop renewable energy alternatives, preferably having a smaller environmental impact than fossil fuels [1]. Biodiesel shows potential as such a renewable liquid fuel for the transport sector, being non-toxic and reducing the emission of most environmentally aggressive components in comparison to petrodiesel [2–4]. Biodiesel in the form of fatty acid methyl ester (FAME) is produced on a multi-million tonne-scale annually by a base-catalyzed transesterification process, in which vegetable oils are reacted with methanol, forming FAME and the by-product glycerol [3].

In the scientific literature, an alternative lipase-catalyzed process has been suggested offering low energy consumption, reduced by-product formation and less waste [5]. Immobilizing the lipases on a support simplifies recycling of the catalyst, which is necessary to make the process economically feasible. Biodiesel produced by a lipase-catalyzed process can be considered "green" since such a process conforms to several of the principles of green chemistry, including catalytic conversion, reduced energy use and high product yield. Replacing methanol with ethanol makes the biodiesel even better from an environmental perspective since, in contrast to methanol, ethanol can be obtained from renewable agricultural products via fermentation [6]. Further, due to the higher molecular weight of ethanol compared to methanol, less oil is used to produce one litre of ethyl ester (FAEE) compared to producing one litre of methyl ester (FAME) [7]. This translates into both economic and sustainability advantages.

As a by-product of biodiesel, glycerol accounts for 10 wt% of the final product. It has become common knowledge that glycerol has a negative effect on lipase activity and stability likely by being adsorbed onto the support of the immobilized lipases and reducing the diffusion of the hydrophobic substrate to the active site of the lipase [8]. This undesirable effect of glycerol will greatly shorten the operational stability of the expensive catalyst and consequently influence the economic viability of the process. In addition, the recovery of glycerol is also made more difficult by its affinity for immobilized lipase. Since the glycerol issue could increase the production cost and affect the process design, it needs to be taken into account when immobilized lipases are used for large scale biodiesel production. This is particularly the case with ethanol-containing azeotropic water concentrations, since it has been shown that the effects of glycerol are further exacerbated if a certain amount of water and glycerol co-exist in the reaction system [9].

Many researchers have reported the inhibitory effect of glycerol on transesterifications catalyzed by immobilized lipases [8,10], but only a few systematic studies have been published on investigating the inhibitory mechanism. Furthermore, thus far little has been reported on the impact of glycerol in solvent-free lipase-catalyzed

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transesterification of a triglyceride feedstock, its interaction with different catalysts and the implications of these issues for the operation of the biodiesel processes. In this paper we address these issues by investigating the effect of glycerol on both the activity and the stability of the biocatalyst in a solvent-free transesterification system catalyzed by *Thermomyces lanuginosus* lipase immobilized on a hydrophobic resin. To assist the investigation, a simple and straightforward dyeing method was developed.

The negative effect of glycerol makes it desirable to trace it in the reaction system. Although glycerol is immiscible with oil and biodiesel and has a higher density than any other component in the liquid phase of the reaction system, it is difficult to observe the separate glycerol phase in laboratory-scale apparatus because the glycerol-rich phase is relatively small and colorless. A dyeing method is therefore introduced to indicate the glycerol partitioning *in situ*. This method was previously used by Zhou and Boocock to stain the polar phase in base-catalyzed methanolysis, ethanolysis and butanolysis to visualize phase behavior [11]. In the transesterification catalyzed by immobilized lipase, the catalyst adds a solid phase to the system. To the best of our knowledge, this is the first time that this dyeing method has been implemented in this type of multiphasic system. The dyeing method is used here to visualize the glycerol in the reaction system and the interaction between glycerol and several different catalysts and support materials.

2. Materials and methods

2.1. Materials

Rapeseed oil and rapeseed derived biodiesel (fatty acid methyl esters) were kindly donated by Emmelev A/S (Otterup, Denmark). Absolute ethanol (≥99.9%) was purchased from Fluka (Buchs, Switzerland). n-Hexane (≥97.0%), cyclohexane (≥ 99.7%) and tert-butyl methyl ether (≥99.8%) were purchased from Sigma–Aldrich (Steinheim, Germany) as HPLC grade. Amaranth, a polar food-grade pigment was also purchased from Sigma–Aldrich (Steinheim, Germany).

The silica, polystyrene, polymethylmethacrylate (PMMA) and polypropylene supports were supplied by Novozymes A/S. All of the catalysts used in this study were kindly donated by Novozymes A/S (Bagsværd, Denmark): Novozym 435 (N435), which is *Candida antarctica* lipase B (CALB) immobilized on a macroporous divinylbenzene-crosslinked polymethylmethacrylate (PMMA); Lipozyme TL IM, which is *Thermomyces lanuginosus* lipase (TLL) immobilized on silica; Lipozyme TL HC, which is TLL immobilized on a polymeric resin (an experimental catalyst).

2.2. Methods

2.2.1. Ethanolysis of rapeseed oil without agitation

The reaction mixture was composed of 10 g rapeseed oil, 5% catalyst (Lipozyme TL IM, N435 or Lipozyme TL HC) and 1.8 mL absolute ethanol, which is 1.0 molar equivalent (eq.) to the total fatty acids in the oil. Reactions were performed in 25 mL glass tubes without any agitation maintained at 35 °C. Samples (30 μL) were taken by a pipette for HPLC analysis.

2.2.2. Ethanolysis of rapeseed oil with agitation

The reaction mixture was composed of 2 g rapeseed oil, 5% catalyst (Lipozyme TL HC) and 0.36 mL absolute ethanol, i.e. 1.0 molar equivalent (eq.) to the total fatty acids in the oil. Reactions were performed in 4 mL vials at 35 °C in a thermo-mixer at a mixing speed of 1000 rpm. Samples (30 μL) were taken by a pipette for HPLC analysis.

2.2.3. Effect of glycerol on catalysts

Varying amounts of glycerol (0–1.0 g) were added into mixtures of 10 g rapeseed oil and 0.5 eq. EtOH in a 25 mL glass tube. The tubes were shaken until the glycerol was dispersed as fine droplets in the oil. Finally, 5 wt% Lipozyme TL HC was added to start the reactions. A further 0.5 eq. EtOH was added after 2 h.

2.2.4. Stability test of catalysts

The ethanolysis reaction was catalyzed by 5 wt% Lipozyme TL HC and was repeated for three batches. The oil phase was decanted after each batch (24 h) and then fresh oil and 1.0 eq. EtOH was added to start a new batch.

2.2.5. Solubility test of dye

Mixtures were prepared in 25 mL glass tubes. Mixture (a) was composed of 10 g rapeseed oil, 1.0 eq. EtOH, 5 mg dye and 5 wt% support, immobilized catalyst or glycerol. Mixture (b) was composed of 10 g biodiesel, 1.0 eq. EtOH, 5 mg dye and 5 wt% support, immobilized catalyst or glycerol.

2.2.6. Affinity test

The catalyst or support (0.5 g) was incubated with 1.0 g glycerol and 5 mg dye for 3 h in a 25 mL glass tube before 10 g rapeseed oil or rapeseed derived biodiesel was added and then mixed by shaking. The mixture was subsequently photographed. Alternatively, 5 mg dye was added to the reaction mixture described above (ethanolysis of rapeseed oil). The mixture was photographed every 2 h for the first 12 h and also after 24 h reaction.

2.2.7. Photography

The glass tubes were laid horizontally on the table with a white background. A normal digital camera (Coolpix S5, Nikon, Japan) was used to take tube photos. Some samples were transferred from the glass tube to a Petri dish for microscope photography (Leica MZ12 equipped with PixeLINK digital camera, Germany).

2.2.8. HPLC analysis of reaction mixtures

Samples (30 μL) were dissolved and diluted in cyclohexane to 1.0 mL and further diluted 100-fold in cyclohexane to achieve concentrations of around 0.25 mg/mL. 10 μL of the solution was injected on an HPLC (Dionex A/S, Hvidovre, Denmark) for analysis of the composition of FAEE, TAG, FFA, DAG and MAG. The HPLC was equipped with a U3000 autosampler, TCC-3000SD column oven and U3400A quaternary pump modules. A Varian ELSD (380-LC) was used for detection with nebulizer and evaporator temperatures of 35 °C and 40 °C, respectively, and a nitrogen flow rate of 1.6 standard liters per minute. The separation was done on a 250 mm × 4.0 mm cyanopropyl column from Sigma–Aldrich (Discovery® Cyano) with an accompanying guard column (20 mm × 4.0 mm) of the same stationary phase and a flow rate of 0.75 mL/min. Program control, data acquisition, and analysis were carried out using Chromeleon 6.8 software. A binary gradient program was applied using phase A: 99.6% hexane, 0.4% acetic acid; and phase B: 99.6% methyl-tert-butyl ether, 0.4% acetic acid.

3. Results and discussion

3.1. Effect of glycerol on immobilized lipase

3.1.1. Effect of glycerol on the activity of immobilized lipase

The effect of glycerol on the activity of immobilized lipase in the ethanolysis of rapeseed oil was studied by addition of external glycerol to the reaction mixture, with the results shown in Fig. 1A. In this case the catalyst (Lipozyme TL HC) lost most of its activity with added glycerol between 2 wt% and 10 wt% compared to the control reaction. Hence, the glycerol greatly inhibited the catalyst. Even a

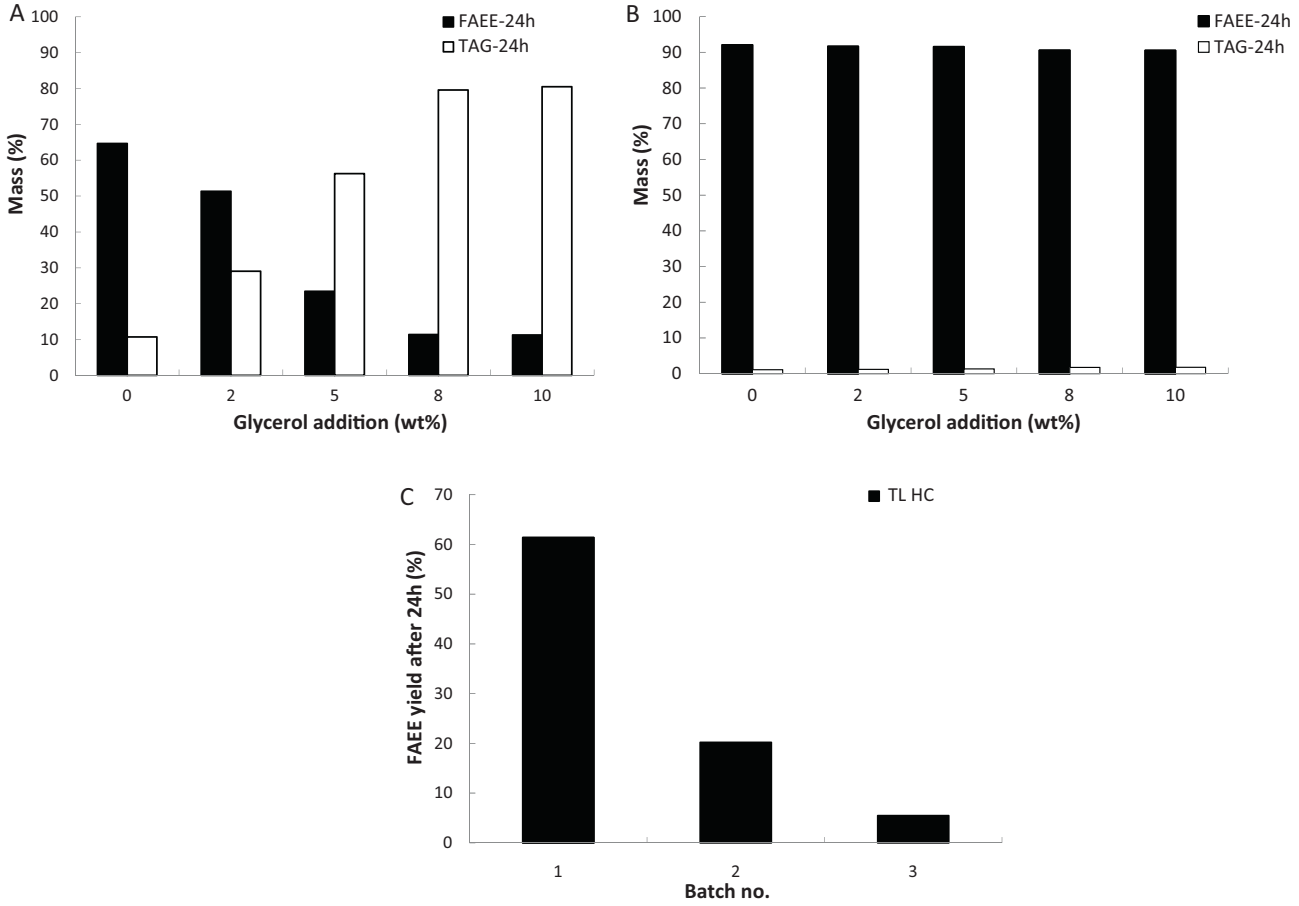

Fig. 1. Effect of glycerol co-existence on the activity and operational stability of Lipozyme TL HC in reactions. Activity without agitation (A), activity with agitation (B) and stability without agitation (C). Amount of glycerol is calculated as percent to the oil (w/w).

small amount of glycerol (2%) reduced the final conversion by 21% in the absence of agitation. It was observed that the immobilized catalyst was aggregated by the viscous glycerol and the liquid phase was opaque throughout the 24-h reaction.

It is clear that the catalyst deactivation is associated with glycerol encapsulating the particles of immobilized TLL, but there are at least three possible mechanisms that might explain the dramatic activity drop of the immobilized lipase. First the diffusion of the substrate to the lipase could be limited by a glycerol layer blocking the pores of the support. A second explanation might be that the hygroscopic nature of glycerol causes a reduced water activity in the system, affecting the performance of the enzyme. These two hypotheses have previously been proposed and studied [8]. A third possibility raised in this study is that glycerol competes with the alcohol substrate to be bound to the active site. In the study by Dossat et al., the inhibition effect was investigated in an esterification reaction system using n-hexane as co-solvent. Conditions were employed that simulated an immobilized catalyst (lipase *Rhizomucor miehei* on Duolite) covered by either glycerol; water; or glycerol maintained with optimal water activity, respectively, and it was concluded that the inhibitory effect on lipase was mainly due to mass transfer limitation caused by glycerol clogging.

To determine the dominant mechanism of glycerol inhibition in solvent-free transesterification where glycerol is also formed as a by-product, an experiment with added glycerol was repeated in a well-mixed reaction system. When sufficient agitation was used, the glycerol could be stripped from the catalyst instantaneously, resulting in no bound glycerol and therefore no catalyst clogging. The result in Fig. 1B shows that when the catalyst clogging by glycerol is avoided, the added glycerol had little effect on the activity of the catalyst and a conversion of 90% was achieved with varying additions of external glycerol. Likewise this indicates that the impact of glycerol as a competing substrate is negligible when glycerol and ethanol coexist. Furthermore, the dehydrating effect of glycerol on lipase on account of its hygroscopic nature is apparently also insignificant. This experiment thus verifies that the mechanism of inhibition suggested by Dossat et al., namely that the inhibition is caused by glycerol clogging the pores of the catalyst, is dominating in solvent-free transesterification as well. This phenomenon is visualized and confirmed by the dyeing method introduced in the following sections.

3.1.2. *Repeated use of Lipozyme TL HC in non-agitated reactions*

The clogging effect of glycerol on Lipozyme TL HC was further investigated by reusing the catalyst for transesterification in a non-agitated reaction system. The activity dropped dramatically from batch to batch and only 5.5% was left by the third batch, as shown in Fig. 1C. Analysis of the reaction data from the first batch indicate that the glycerol produced in the first batch corresponds to 4.7 wt% of the reaction mixture. The yield of FAEE in the subsequent batch dropped to 21%, which is very close to the 23% yield that was achieved in a standard, non-agitated reaction when 5 wt% external glycerol was added (Fig. 1A). This experiment illustrates that a process using Lipozyme TL HC for transesterification of glycerides must be designed to ensure removal of glycerol from the catalyst, for example by applying agitation, if operational stability is to be

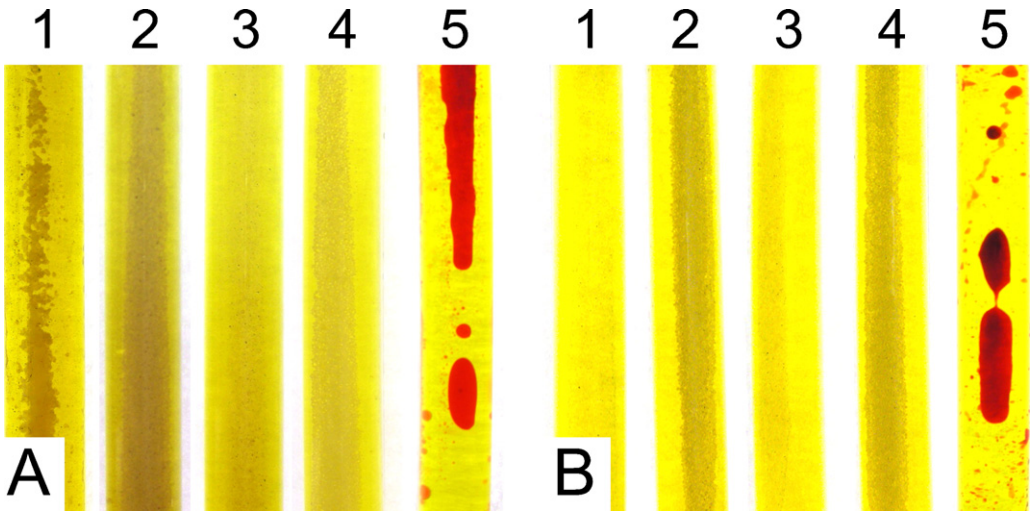

Fig. 2. The solubility of dye in rapeseed oil based mixture (oil, 1.0 eq. EtOH and dye) (A) and biodiesel-based mixture (biodiesel, 1.0 eq. EtOH and dye) (B). The solids in tube 1 – silica support, tube 2 – N435, tube 3 – Lipozyme TL IM, tube 4 – Lipozyme TL HC, tube 5 – glycerol.

achieved. Indeed, repeated use of catalyst in the agitated reaction system (used for the results shown in Fig. 1B) consistently resulted in final conversions above 90% (data not shown).

3.2. Dyeing method to indicate the effect of glycerol

3.2.1. The solubility of the dye in simulated reaction mixtures

Before the dye was introduced to indicate glycerol partitioning in the biodiesel system, its physical chemistry was assessed by simple experiments. The dye was purchased in the form of a dry powder. It is polar and consequently has no solubility in rapeseed oil or biodiesel. Instead, it exhibits great solubility in glycerol and limited solubility in anhydrous ethanol. Fig. 2A shows pictures of the dye in a solution of oil and 1.0 molar equivalent EtOH. To these mixtures were added either solid particles (hydrophilic silica support or one of three immobilized lipases: N435, Lipozyme TL IM or Lipozyme TL HC) or glycerol (10 wt%). Dye was added in an amount sufficient to stain all of the glycerol that would be produced if the oil was fully converted into biodiesel. Fig. 2B shows pictures of similar dye mixtures where the oil has been replaced with biodiesel. Both figures demonstrate that the dye could not be dissolved in either oil or biodiesel mixtures and that the dye also has no influence on silica or immobilized lipases. On the other hand, the same amount of dye powder was easily dissolved in glycerol, which was then stained red. The presence of 1.0 molar equivalent ethanol in the oil did not affect the ability of the dye to work as an indicator in these systems.

3.2.2. Affinity of the glycerol byproduct for immobilized lipases in ethanolysis

3.2.2.1. Immobilized lipases in ethanolysis. The glycerol affinity for immobilized lipase was investigated in enzyme-catalyzed ethanolysis reactions. Reactions took place at 35 °C with 5% catalyst loading, 1.0 molar equivalent of anhydrous ethanol for 24 h without any agitation. The photos in Fig. 3 show the partitioning of glycerol during the ethanolysis in this multiphasic system. As can be seen in Fig. 3A, glycerol formed a layer on the surface of Lipozyme TL IM particles. The liquid phase was clear with no free glycerol being released from the catalyst to the bulk solution, suggesting that the formed glycerol was fully adsorbed on/in the Lipozyme TL IM particles.

The affinities of N435 and Lipozyme TL HC for glycerol are less than that of Lipozyme TL IM, as can be seen in Fig. 3B and C. Part of the produced glycerol remained on the surfaces of both catalysts and some free glycerol droplets are noticeable in the oil phase of each reaction. It was also observed that the aggregation of Lipozyme TL HC was more severe than that of N435 (photo not shown), which can probably be explained by more glycerol being produced by the former catalyst. In order to test this hypothesis, the activities of N435 and Lipozyme TL HC were compared (see Fig. 4A). Under the given conditions N435 had a lower activity than Lipozyme TL HC (determined by HPLC analysis) and consequently produced less glycerol.

The difference in substrate specificity between CALB (the lipase on N435) and TLL (the lipase on Lipozyme TL IM and Lipozyme TL HC) is illustrated by comparing the consumption of TAG as a

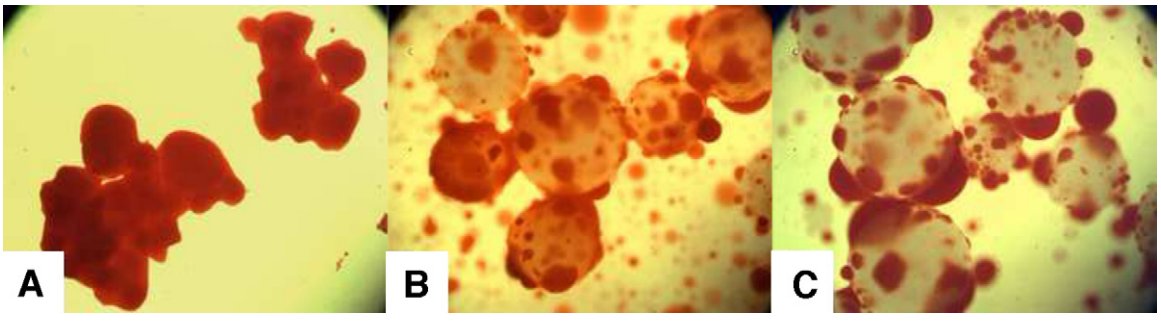

Fig. 3. Glycerol partitioning in ethanolysis of rapeseed oil, catalyzed by different immobilized catalysts. Lipozyme TL IM (A), N435 (B) and Lipozyme TL HC (C).

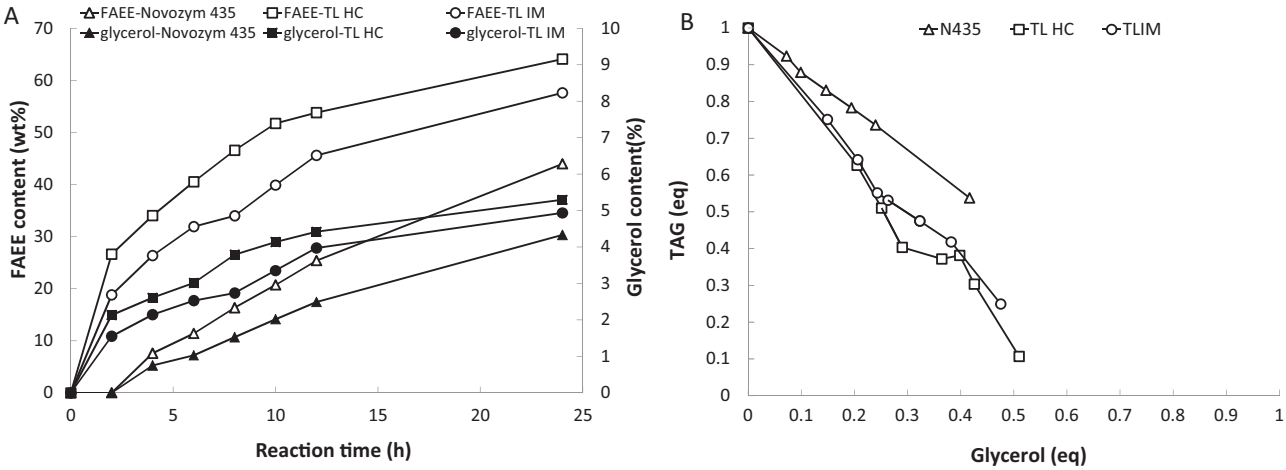

Fig. 4. Reaction profiles of N435, Lipozyme TL HC and Lipozyme TL IM. FAEE and glycerol production as function of time (A); TAG consumption as a function of glycerol production (B).

function of the production of glycerol from the three catalysts, shown in Fig. 4B. The TAG consumption curves of Lipozyme TL IM and Lipozyme TL HC are steeper than that of N435, indicating that TLL consumed more TAG than CALB to produce the same amount of glycerol. In other words, TLL worked better with TAG than with DAG and MAG, which is most likely related to its 1,3-positional specificity reported by many researchers [12,13]. DAG and MAG turned out to be the favorite substrates for CALB which is less regiospecific. This substrate specificity is likely due to the different structures of active sites. The elliptical, steep funnel-like binding site of CALB has limited space for hosting substrates [14]. TLL, structurally homologous to lipase from *Rhizomucor miehei*, has a hydrophobic crevice-like binding site with a larger space available to bulkier substrates [14,15].

3.2.2.2. Glycerol production and accumulation on the catalysts. Finally, Fig. 5A and B shows the glycerol accumulation on Lipozyme TL IM and N435, respectively, as a function of the reaction time.

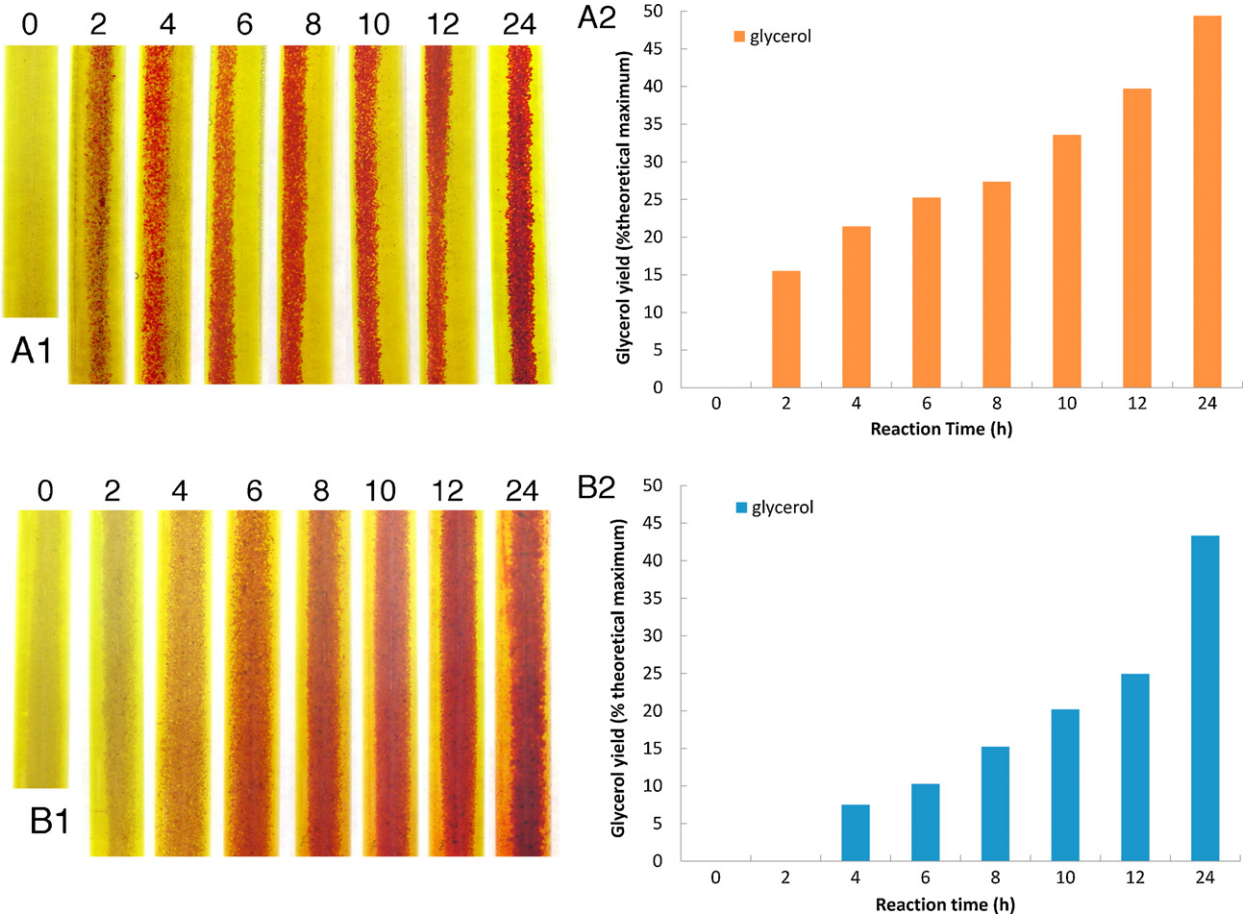

Fig. 5. Glycerol accumulation on catalysts as a function of reaction time. Lipozyme TL IM (A) and N435 (B).

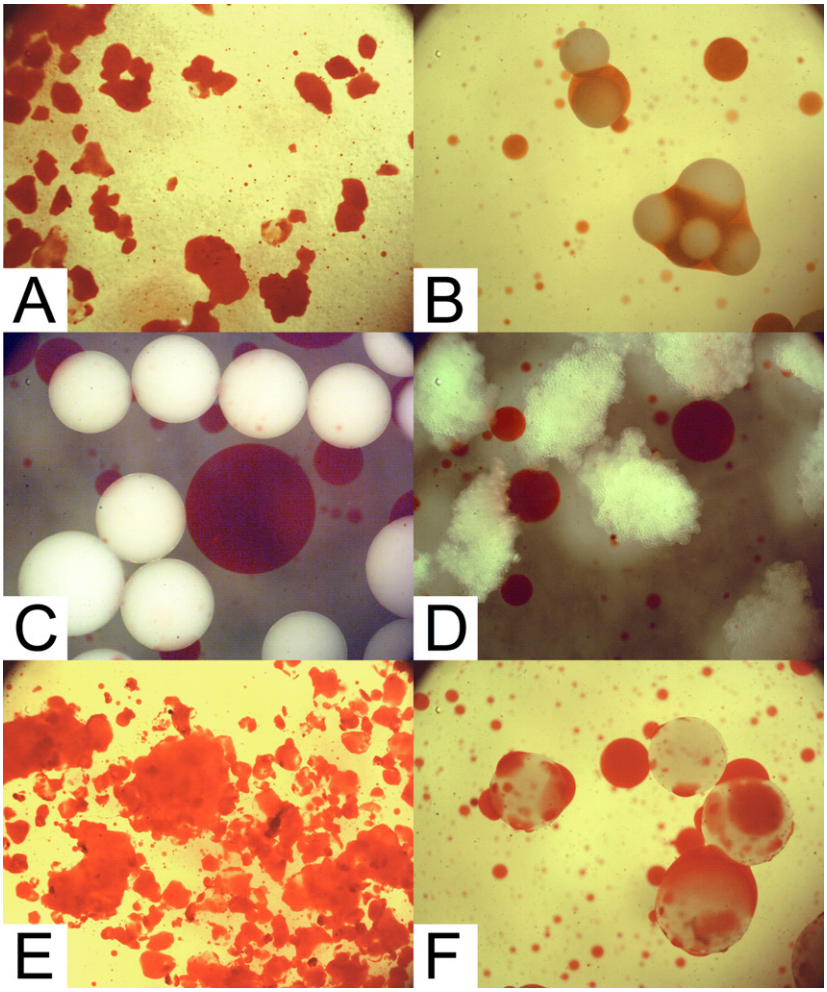

Fig. 6. Affinity of glycerol for supports and immobilized lipases. The supports shown in the photos are: silica (A), polystyrene (B), polymethylmethacrylate (PMMA) (C) and polypropylene (D). The immobilized lipases are: Lipozyme TL IM (E) and N435 (F). In each photo, the particles of supports and immobilized lipases are in white and the glycerol droplets are in red (in the printed version of this article, white and red are shown as white and black, respectively).

As the reaction proceeded from 0 h to 24 h, glycerol was produced and most of it adhered to the catalyst, particularly in the case of Lipozyme TL IM. With dye present in the reaction, the accumulation of glycerol could be followed by the intensity of the red color and correlated with the glycerol production curve (the red color appears as darker/black areas in black and white print). Each data point on the glycerol production curve was calculated from the compositions of FAEE, FFA, TAG, DAG and MAG at the corresponding time point, as measured by HPLC.

The color of the catalyst (N435 or Lipozyme TL IM) became more intense as more glycerol was produced, suggesting that the dyeing method could be developed into a tool for measuring the glycerol production by quantifying the color intensity. It was observed that glycerol began to form earlier in reactions catalyzed by Lipozyme TL IM than those by N435. The color could be detected after only 2 h in the TL IM-catalyzed reactions, whereas the color was not obvious until after 4 h in N435-catalyzed reactions as seen from the photos in Fig. 5A-1 and B-1. This correlates well with the data presented in Fig. 4A.

3.2.3. *Affinity of glycerol for immobilized lipases and supports in simulated mixtures*

Four supports were tested individually for their affinity for glycerol in a simulated system based on a synthetic mixture of glycerol, dye and oil. The results are shown in Fig. 6. The silica (Fig. 6A) was immediately covered by the glycerol, indicating that it has a great affinity for glycerol due to the hydrophilic properties of the material. No free glycerol droplets could be seen in the oil, implying that 0.5 g silica could adsorb at least 1.0 g glycerol, in agreement with the observation in silica-based TL IM-catalyzed-reaction. This was further verified in a separate experiment, where it was observed that 1.0 g silica could adsorb as much as 2.0–3.0 g glycerol (data not shown). The polystyrene support (Fig. 6B) also exhibited some affinity for glycerol in that the particles were aggregated by the glycerol, forming clumps. On the other hand, the polymethylmethacrylate (PMMA) support and the polypropylene support, shown in Fig. 6C and D, respectively, both appear to be very hydrophobic in that no glycerol was adsorbed on these materials. In these cases the free glycerol droplets were dispersed in the oil.

Immobilized lipases (Lipozyme TL IM and N435) were also tested for their affinity for glycerol using the same method. As expected, Lipozyme TL IM was immediately covered by the glycerol (Fig. 6F) just as in transesterifications carried out by this catalyst. Similar results were obtained with silica without enzyme. The effect of the enzyme on the affinity for glycerol was evident when the support of PMMA and its immobilized lipase (N435) were compared (Fig. 6C and F). The PMMA support is hydrophobic enough to avoid being covered by the glycerol even after incubation with added glycerol for 3 h at room temperature, whereas N435

attracted some glycerol onto its surface during incubation. This implies that the immobilization of lipase onto the support makes it less hydrophobic, possibly due to polar groups on the protein. More importantly, it also shows that it is not sufficient to test the support alone when investigating the glycerol affinity of an immobilized catalyst.

4. Conclusions

The glycerol formed in biodiesel synthesis by immobilized lipases can severely reduce the reaction rate by surrounding the catalyst in a hydrophilic layer, thereby limiting the mass transfer of substrate to the enzyme.

The developed dyeing method successfully visualizes this interaction between glycerol and lipase catalysts, showing extensive interaction between glycerol and a hydrophilic catalyst. Conversely, hydrophobic catalysts are able to release most of the glycerol produced, especially when agitation is applied.

Similar results can be obtained with simulated reaction mixtures, suggesting that the dyeing method could have great potential when screening enzyme supports for materials applicable to biodiesel production.

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PAPER 2

Enzymatic biodiesel production in a stirred tank reactor

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Status: manuscript

Enzymatic biodiesel production in a stirred tank reactor

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Abstract:

Solvent-free immobilized lipase (NS 88001)-catalyzed biodiesel production by ethanolysis of rapeseed oil has been studied in a 150 mL stirred-tank reactor (STR) within a power input per volume of 1.0 W/L close to industrial application. The scale up of the process was carried out in a 20 L STR (10 L working volume). Both STRs were studied in batch mode with respect to the mixing related mechanical stability of NS 88001 and external mass transfer effect on the activity and operational stability of NS 88001. The mass transfer could not be significantly improved by increasing the stirring speed when it is already above the just suspended speed (Njs). The mass transfer limitation was significant at 10 wt% loading of NS 88001.

A similar catalyst performance was obtained in the pilot-scale STR in terms of initial rates and reaction progress, indicating that the catalyst performance at a scaled STR can be accurately predicted by that in a smaller STR. Additionally, high stability of NS 88001 was observed with 96% ethanol in both scales, being repeatedly used without apparent activity loss for at least 5 batches in the lab-scale STR and 4 batches in the pilot-scale STR.

Mechanical stability of NS 88001 has also been investigated in both lab- and pilot-scale STRs with a testing period of 120 hours, using particles pre-sieved to a minimum size of 500 μm. Above 80% (lab scale) and 74% (pilot scale) of the particles remained larger than 500 μm after testing at a P/V that can be useful for an industrial application (1.0 W/L) and 95% (pilot scale) was obtained at a minimum P/V (0.2 W/L) corresponding to Njs. The size distribution of broken particles was found to be large enough not to cause clogging of the filter used to retain the catalyst in the reactor.

Keywords: immobilized lipase, biodiesel, stirred tank reactor, scale up

1. Introduction

Biodiesel has been developed as a potential energy alternative to traditional fossil fuels. Conventional biodiesel, also known as fatty acid methyl ester (FAME) is produced from virgin vegetable oil and methanol via an alkali-catalyzed reaction. To further reduce the dependence on fossil fuel, bioethanol can replace methanol since methanol is generally produced from natural gas while ethanol can be obtained from biomass via fermentation (Al-Zuhair, 2007). As an alternative to the chemical reaction, lipase-catalyzed transesterification has attracted significant attention recently since it has the advantages of low energy consumption, reduced formation of by-products and waste (Nielsen et al., 2008).

In order to make the synthesis of biodiesel economically viable, effective use of the lipase demands reuse via for example immobilization of the enzyme on a support, which also simplifies the downstream processing of the product.

One of the characteristics of the solvent-free immobilized lipase-catalyzed biodiesel reaction is the multi-phasic nature of the reaction system composed of solid particles of immobilized lipase, a lipid phase and additionally a polar phase when alcohol exceeds the solubility limit (or when the by-product glycerol is present). Sufficient agitation is required to reduce the diffusion boundary layer from liquid to the solid surface and increase the interfacial area by creating the emulsion of the partial glycerides with ethanol. It is also necessary to remove the products from the surface of the immobilized lipase, especially by-product glycerol, which can have a negative effect on immobilized lipases by forming a hydrophilic layer on the surface of the catalyst (Xu et al., 2011).

Stirred tank reactors (STRs) are the most often-used reactors for biocatalysis at different scales because of the ease of construction, operation and maintenance (Balcáo et al., 1996). However, most reported lipase-catalyzed biodiesel studies have so far been carried out in shaking flasks at modest scales. There are few reports regarding the enzymatic production of biodiesel in STRs from a reactor perspective. Many applications of immobilized lipases in STRs are found in the production of higher-value and viscous lipid products, such as lubricants (ester oils) and margarine fats (Keng et al., 2008; Zhang et al., 2001).

One of the potential disadvantages of STR is the mechanical damage to the immobilized enzyme by the rotating impellers. The damage to the biocatalysts can reduce the catalyst reusability affecting the economic feasibility of the processes. Also any debris can potentially contaminate the product (Halim et al., 2009; Tufvesson et al., 2010). Although it is an

important factor to the application of STRs for immobilized lipase-catalyzed reactions, very few reports have investigated the mechanical stability of immobilized lipases catalyzing the biodiesel reaction in an STR.

Reports of the scale-up of the immobilized lipase-catalyzed biodiesel process are rare, partly because of the scale-up challenges associated with the complex reaction system. Reduced productivity and performance are often encountered with the scale-up of bioreactors as a result of lower mixing efficiency and altered hydrodynamic conditions (Doran, 1995). For industrial implementation of enzymatic biodiesel, a study is necessary to identify the critical parameters of the process and predict the biocatalyst performance at a larger-scale.

This work focuses on solvent-free immobilized lipase-catalyzed ethanolysis of rapeseed oil for FAEE-biodiesel production in STR and its scale up. Lab-scale experiments were designed based on a scale-down methodology (Doran, 1995), the conditions of which were determined based on a realizable and economic power input on an industrial scale (< 1.0 W/L). In this way, the mixing conditions of the large-scale STR could be simulated in the lab-scale STR (150 mL). A pilot-scale STR (10 L) was designed to verify this approach at a larger scale. Comprehensive studies have been carried out in lab- and pilot-scale STRs with respect to the effect of external mass transfer on the catalyst performance, e.g. activity and operational stability, as well as the effect of mixing on the mechanical stability of the immobilized lipase.

2. Materials and methods

2.1 Materials

Rapeseed oil and rapeseed oil-derived FAME-biodiesel were kindly donated by Emmelev A/S (Otterup, Denmark). Absolute ethanol (≥ 99.9%) was purchased from Fluka Chemie (Buchs, Switzerland). n-Heptane (≥99%) and t-butyl methyl ether (≥ 99.8%) were purchased from Sigma–Aldrich (Steinheim, Germany) as HPLC grade. The experimental biocatalyst, NS 88001, used in this study was kindly donated by Novozymes A/S (Bagsværd, Denmark), which is *Thermomyces lanuginosus* lipase (TLL) immobilized on a polymeric resin.

2.2 Reactor configurations

The lab-scale STR set up comprises three identical baffled glass tanks (250 mL with 150 mL working volume). A four-blade marine propeller is implemented in each and driven by the

same motor to create a down-flow. The schematic drawing of the STR is shown in Figure 1 (left) and the dimension is marked in the figure. The main body of the setup is submerged in a water bath for maintaining the temperature required for reactions.

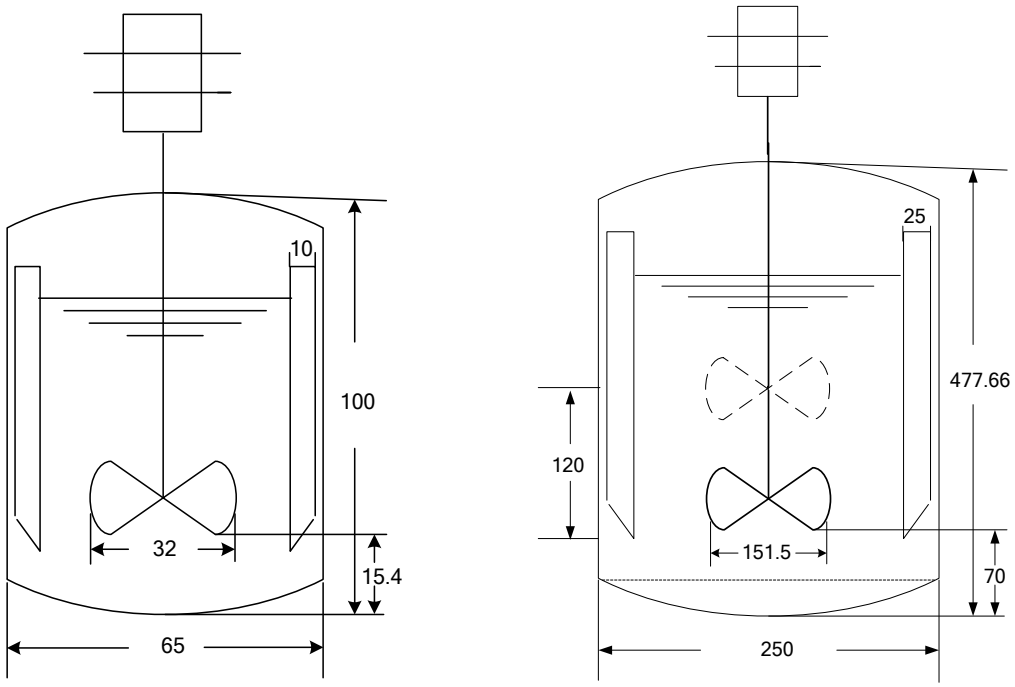

Figure 1 Sketches of STRs (left-lab-scale, right-pilot-scale; unit in mm)

2.3 Ethanolysis of rapeseed oil in lab-scale STR

The reaction mixture in lab-scale STR was composed of 90 g rapeseed oil, 2.5-10 % catalyst (NS 88001) and 9-27 mL absolute ethanol or 96% ethanol, which is 0.5-1.5 molar equivalent (eq) to the total fatty acids in the oil. The total amount of ethanol was added in three different ways: one-step at the beginning, in three equal portions (0.5 eq) at 0, 2 and 4 hours reaction time or continuously added within the first 2 hours. The applied stirring speeds were within 500 rpm-1200 rpm corresponding to approx. 0.1-1.7 W/L. Reactions were performed at 35 ^{o}C for 24 hours. Samples (50 µL) were taken at regular intervals for HPLC analysis.

2.4 Operational stability test of NS 88001in lab-scale STR

The ethanolysis reaction was catalyzed by 5 wt% NS 88001 and 1.0-1.5 eq ethanol (absolute or 96%) was added in a one-step or stepwise manner, as described in section 2.3. The stirring speed was tested from 800-1200 rpm. The oil phase was decanted after each batch (24 hour)

and then fresh oil and EtOH was added to start a new batch. Likewise the batch reaction was repeated for five times.

2.5 Mechanical stability of NS 88001 in lab-scale STR

The catalyst particles of size above 500 μm pre-sieved by a sieve of mesh size 500 μm were used in mechanical stability tests and they were tested at the same reactor loading, approx. 10 % by volume, at three different conditions as follows: a. in 5 repeated batch reactions as described in section 2.3; b. rapeseed oil derived FAME-biodiesel; c. a mixture of rapeseed oil and glycerol (2:1 w/w). The catalyst particles were stirred for 120 hours at each condition.

After the tests the catalyst particles were separated and washed by an equivalent volume of 2-propanol to double volumes of particles. They were then washed by the same volume of cyclo-hexane and left to dry in a fume hood. The dried particles were sieved again for measuring the fraction of particles above 500 μm.

2.6 Ethanolysis of rapeseed oil in pilot-scale STR

The scaling up of the ethanolysis of rapeseed oil was carried out in a 20 L pilot-scale STR equipped with four baffles. The agitation shaft was designed to be able to accommodate one or more impellers at flexible positions. Two types of impeller, marine propeller (D 151.5 mm) and Rushton turbine (D 120 mm), were tested respectively using stirring speeds within 80 -270 rpm. The geometric details of the reactor are shown in Figure 1. A filter with mesh size 36 μm was installed near the bottom of the tank to retain the catalyst particles. The reaction mixture was composed of 9 kg rapeseed oil, 450 g NS 88001 (5 wt%) and 2.7 L 96% ethanol corresponding to 1.5 eq, or 50 % stoichiometric excess. The ethanol was continuously added to the reactor within the first 2 hours. Reactions were performed at 35 ^{o}C for 6 hours, with samples taken at regular intervals for HPLC analysis.

2.7 Operational stability test of NS 88001in pilot-scale STR

The ethanolysis described in section 2.6 was carried out at 250 rpm with a single propeller. The oil phase was drained after 6-hour reaction and the catalyst particles were kept in the reactor with fresh oil overnight. The oil was drained on the second day and fresh oil and ethanol were added to start a new batch. Four consecutive batches were conducted with the same catalyst loaded in the tank.

2.8 Mechanical stability test of NS 88001 in pilot-scale STR

Mechanical stability was evaluated by first carrying out a reaction according to the method described in section 2.6, followed by continued stirring of the catalyst particles in the reaction mixture for 120 hours. One single propeller or dual propellers were used in the experiments and stirring speeds were adjusted to create similar power inputs as used in lab-scale experiments. Particle samples (approx. 10 g) were taken to be washed and dried with the same procedure as mentioned in section 2.5 before the measurement of particle size distribution. This was carried out on a Malvern Mastersizer 2000, equipped with a Hydro S sample dispersion unit. Samples (0.2-0.3 g) were suspended in 20 mL isopropanol prior to analysis. All particle size determinations were carried out by Novozymes A/S (Bagsværd, Denmark).

2.9 HPLC analysis of reaction mixtures

Samples (50 μL) were dissolved and diluted in a mixture of 0.5 mL acetic acid and n-heptane (4:1000, v/v) and further diluted 100-fold in the same mixture to achieve concentrations of around 1.0 mg mL^{-1}. 40 μL of the solution was injected on an HPLC (Dionex A/S, Hvidovre, Denmark) for analysis of the composition of FAEE, TAG, FFA, DAG and MAG. The HPLC was equipped with U3000 autosampler, TCC-3000SD column oven and U3400A quaternary pump modules. A Corona® Charged Aerosol Detector (Thermo Scientific Dionex, Chelmsford, MA, USA) was used for detection with nitrogen at an operating pressure 35.0 psi. The separation was done on a 250 mm × 4.0 mm cyanopropyl column (Discovery® Cyano from Sigma–Aldrich, Steinheim, Germany) at a flowrate of 0.75 mL min^{-1}. Program control, data acquisition, and analysis were carried out using Chromeleon 6.8 software. A binary gradient program was applied using phase A: 99.6% n-heptane, 0.4% acetic acid; and phase B: 99.6% t-butyl methyl ether, 0.4% acetic acid (Foglia, 1997).

Yield of biodiesel is defined as FAEE mass percentage in the mixture of FAEE and glycerides including TAG, DAG, MAG and FFA as indicated by the equation below.

$$\text{Yield(mass\%)} = \frac{\text{FAEE}}{\text{FAEE+TAG+DAG+MAG+FFA}} * 100\%$$

3. Results and discussions

3.1 Catalyst loading in lab-scale STR

The initial reaction rate normally increases with the catalyst loading when substrate is excess. However, it does not always increase linearly with the catalyst loading, meaning that the specific activity (rate per g catalyst) is affected by a phenomena such as enzyme deactivation/inhibition or mass transfer limitation. It has been previously investigated that absolute ethanol has a solubility limitation in the vegetable oils, around 0.5 eq; and the excess ethanol can possibly inhibit the enzyme. Therefore, 0.5 eq absolute ethanol was used in this experiment to avoid the disturbance of enzyme inhibition. Table 1 shows the specific activity of NS 88001 decreases as the increasing catalyst loading even though the difference between 2.5 % and 5 % is not remarkable. However, the obvious drop of specific activity was found at 10 % catalyst loading, which can be most likely explained by excessive external mass transfer limitation of substrate at such a high catalyst density. As indicated by equation A.4 in the Appendix, a higher catalyst loading can increase the apparent viscosity of the mixture and consequently reduce the Reynolds number according to equation A.5, indicating less turbulent flow. As a result, the external mass transfer is reduced leading to a less efficient use of catalyst. Although the best specific activity occurs at a catalyst loading of 2.5 %, the space-time-yield is obviously low meaning a low efficiency of utilizing the reactor. Therefore, 5 % catalyst loading is chosen in the following experiments.

Table 1 The specific activities at different catalyst loadings of NS 88001

Catalyst loading (wt %)	2.5	5	10
Specific activity (FAEE %/catalyst loading %/hour)	17.07±2.12	15.13±1.36	10.59±2.27
Space-time-yield (FAEE%/hour/L)	341.5±42.4	581.9±52.4	756.2±161.9

95% confidence intervals are used; reaction conditions: 0.5 eq absolute ethanol, 1200 rpm corresponding to approx. 1.7 W/L

3.2 Just suspended speed

A complete suspension of catalyst is required to ensure a maximum surface area of catalyst particles exposed to the substrates. The complete suspension can be ensured by the stirring speed above ‘just suspended speed (Njs)’ in STR. Njs for NS 88001 at different conditions

has been calculated and the calculated results are compared to the observed results from experiments, as shown in Table 2. Although the calculated results are higher than the observed results, both results are in the same tendency that Njs is reduced as the ethanol loading is increased. A higher ethanol loading can reduce the viscosity of the reaction mixture, which results in a smaller Njs, as suggested by equation A.5. The effect of viscosity on Njs is found to be small in the turbulent regime (Paul et al., 2003). However, the flow patterns at studied conditions are in the transitional flow regime because the Re at Njs are around 400, calculated according to equation A.7. It is therefore reasonable to explain the varied Njs by the viscosity difference.

The equation A.5 has been proved by the visual measurements to be helpful to estimate a sufficient stirring speed at a given condition, especially when the visual measurement is not feasible. It is the case for the nontransparent pilot-scale STR, which is a steel tank. The calculated Njs for the same catalyst of 5 wt% in pilot-scale STR with an initial loading of 0.5 eq 96% ethanol is about 200 rpm, which can be verified by the results of initial rates as a function of P/V in the following section.

Table 2 Comparison of calculated and observed Njs of NS 88001 in the lab-scale STR

Njs(rpm)	96% ethanol addition (eq)	
	0.33	0.5
Calculated	787	780
Observed	700	650

3.3 Initial rate

3.3.1 Effect of power input per volume (P/V)

When a single marine propeller was used in the reaction, the initial rates at both lab- and pilot-scale are compared in Figure 2. The experiments were conducted from observed Njs (650 rpm) in the lab-scale STR, which is corresponding to 0.2 W/L (the power input is calculated according to equation A.6). The initial rates were higher than those in the pilot-scale STR at the similar P/Vs, as Figure 2 shows. It is possibly related to the different ethanol feeding strategies used in the two scale trials, where the ethanol concentration was higher in the beginning of the reaction in the lab-scale STR than that in the pilot-scale STR. It indicates that the continuous feeding strategy needs to be optimized.

The initial rates of both scales slowly increased as the increase of P/V in the range of 0.2-0.65 W/L. It implies at each scale a similar uniformity of catalyst particles was achieved in this range of power input, i.e. the mass transfer efficiency was similar at the tested power inputs.

A great boost of initial rate arose when the P/V was increased from 0.02 W/L to 0.03 W/L in the pilot-scale STR, which suggests a P/V of 0.03 W/L (100 rpm) is sufficient to lift most of the catalyst particles. Therefore, Njs of pilot scale is likely within 100-170 rpm which is a little far from calculated Njs (200 rpm).

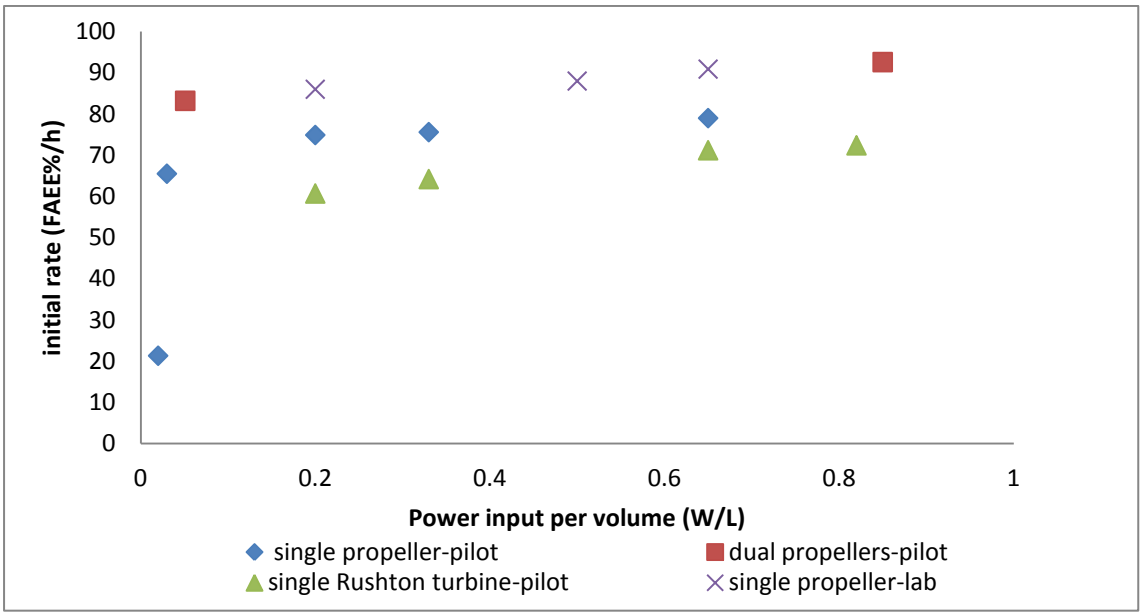

Figure 2 The effect of P/V on the initial rates. Conditions: one-step adding 0.5 eq 96% ethanol in lab-scale trials and continuously adding the same amount of ethanol in pilot-scale trials in the first 20 min of the reactions

3.3.2 Effect of Impeller type and number

Axial flow impellers such as the marine propeller can circulate the flow in an axial direction and are thus efficient at suspending solids, while radial flow impellers such as the Rushton turbine are normally used for liquid-liquid blending because of the higher shear and turbulence levels provided by this type of impeller (Paul et al., 2003). In spite of this, the Rushton turbine is more often used in immobilized lipase-catalyzed reactions than marine propeller in available literatures (Keng et al., 2008; Chaibakhsh et al., 2010). It is probably because this impeller is good at liquid-liquid blending which is advantageous to high viscous reaction mixture.

Both types of impellers have been compared in the scaled enzymatic process and their effects on the initial rate are shown in Figure 2. The initial rate achieved with a single Rushton

turbine was lower than that by a single propeller at the same P/V, which supported that the axial flow impeller is better at solid-liquid reactions than the radial flow impeller. Dual propellers proved even more efficient than one single propeller because a similar initial rate can be achieved with dual propellers at a much lower P/V (0.05 W/L) as shown in Figure 2. It is because the two propellers caused more turbulence than the single one resulting in the improved mass transfer of substrates, which is extremely useful to the reactors of a high ratio of liquid height/tank diameter.

3.4 Reaction progress

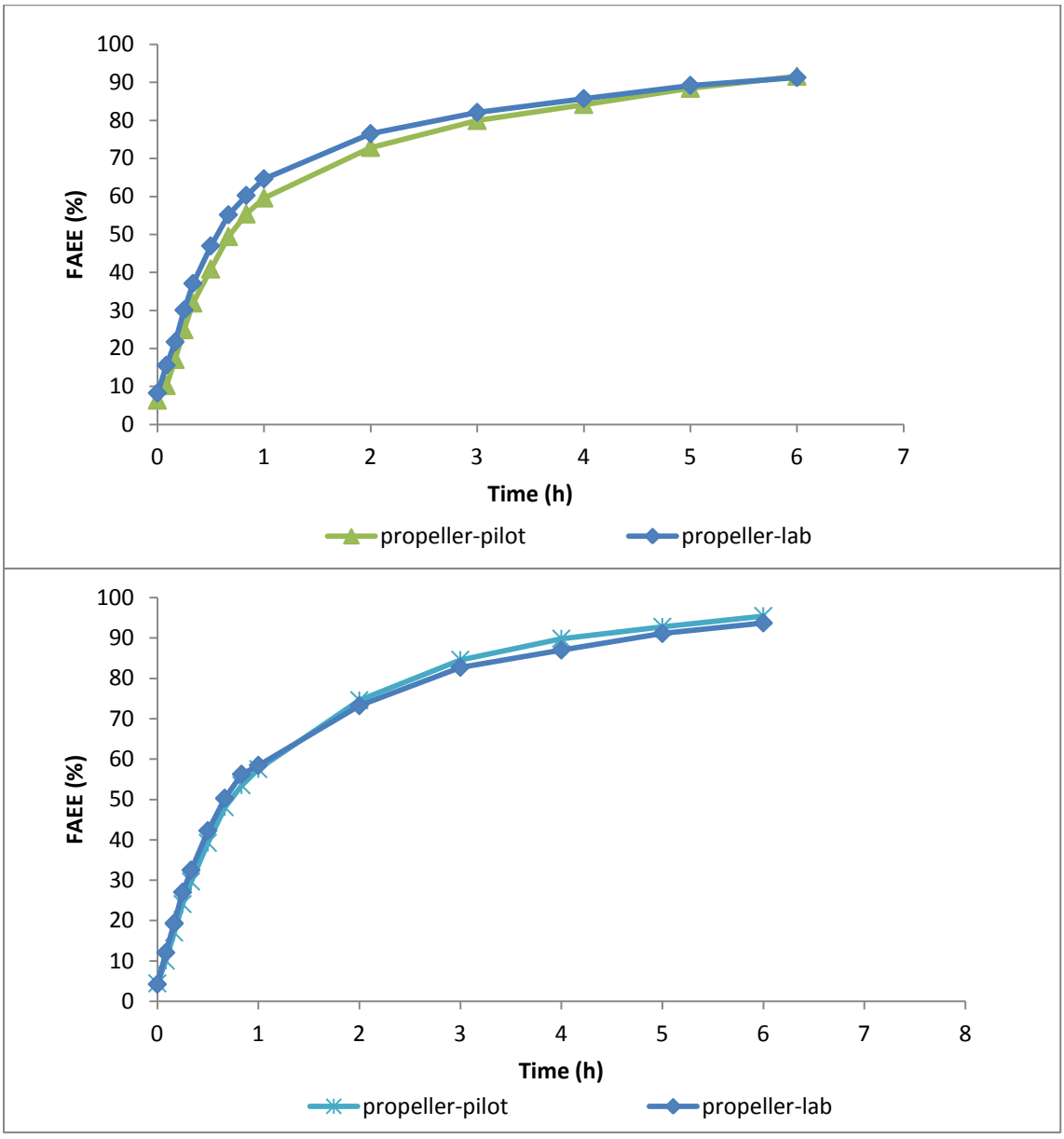

Figure 3 Reaction progresses at different scales 0.2 W/L(top) and 0.65 W/L (below)

Figure 3 compares the time courses of the reaction at the lab and pilot-scale using the same continuous feeding strategy for 1.5 eq ethanol over 2 h at the same P/V (0.2 or 0.65 W/L). In general, the performances of the immobilized lipase at these two different scales are very similar although the reactions at pilot-scale lagged slightly behind those at lab-scale. This is consistent with the initial rate determinations in the previous section, which showed that the mixing was slightly more efficient in the lab scale.

Furthermore, the reaction progresses at 0.2 and 0.65 W/L in both scales are also very close to each other, which indicates a similar mixing effectiveness can be achieved at these two power inputs and it is not necessary to apply higher P/Vs than 0.2 W/L under the studied conditions.

3.5 Operational stability

The productivity of immobilized lipase is very important to the process viability of the enzymatic biodiesel production. A high productivity greatly depends on a good operational stability, meaning the consistent product yield of each batch without altering the reaction time.

Most lipases require an interface of lipid and water to be activated, which is also true for TLL on NS 88001. It is believed that a certain amount of water can enhance the activity and stability of TLL. Therefore, two types of ethanol (absolute ethanol and 96% ethanol) have been used and their effects on the operational stability of NS 88001 have been studied associated with the mixing effect in the lab-scale STR. The results are shown in Figure 4 and 5.

As Figure 4 shows, FAEE yields after 6 hours of five successive batches are stable under the conditions of 1.0 eq absolute ethanol added stepwise at high stirring rates (1000 and 1200 rpm). However, at a lower speed (800 rpm) the final FAEE yield gradually decreased through the batches using the same way of adding the same amount of absolute ethanol. As observed, catalyst particles became heavier due to the glycerol and the stirring speed was no longer sufficient to maintain a complete suspension of catalyst particles. As a consequence, the reaction was slowed down and ethanol accumulated in the reactor, causing deactivation of the lipase.

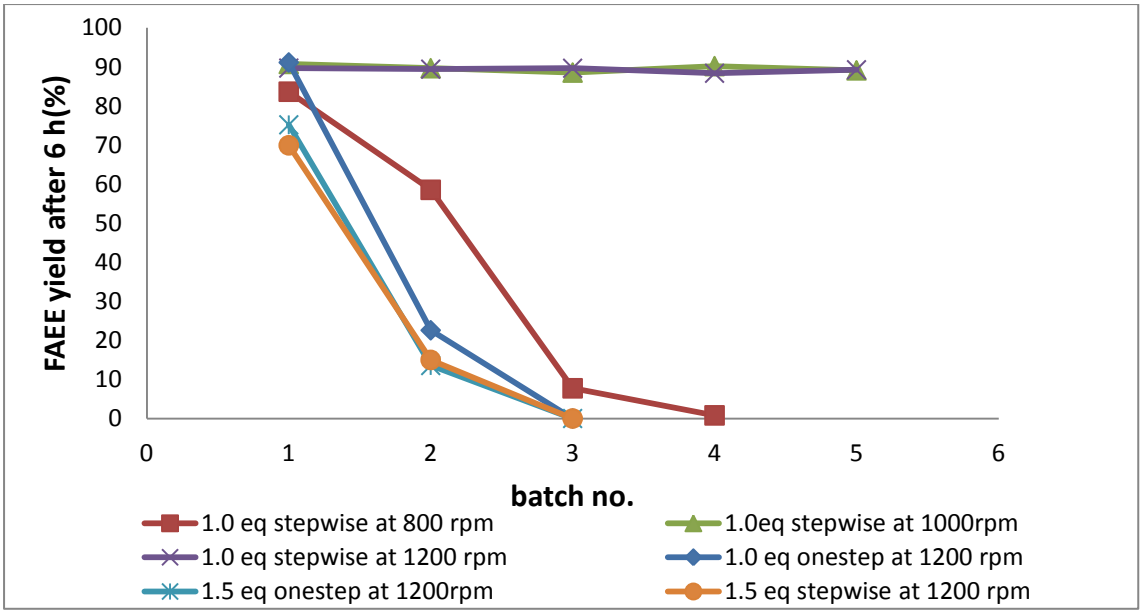

Figure 4 Stabilities of NS 88001 with dry ethanol in lab-scale STR (0.5-1.7 W/L)

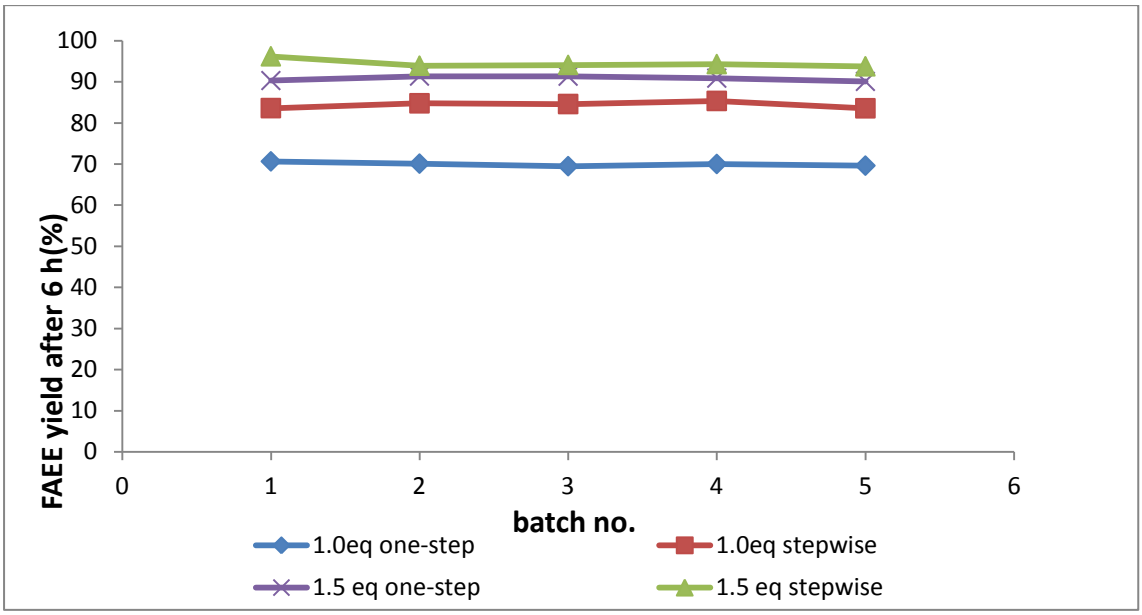

Figure 5 Stabilities of NS 88001 with 96% ethanol in lab-scale STR at 800 rpm (0.5 W/L)

The operational stability of the catalyst is even worse with one-step adding 1.0 eq or 1.5 eq absolute ethanol. Under these conditions the operational stabilities are predominantly affected by the deactivating effect of excessive absolute ethanol.

On the contrary, the catalysts are very stable with 96% ethanol under similar conditions without obvious activity loss during the consecutive 5 batches, as Figure 5 shows about the results from the low stirring speed (800 rpm) corresponding to about a P/V of 0.5 W/L. These

significant improvements on the operational stability of NS 88001 is most likely because the water from the 96% ethanol can not only maintain the desired water activity for lipase, also slightly lower the viscosity of the liquid mixture and increase the hydrophilicity of glycerol droplets, resulting in a lower Njs and a more efficient releasing of glycerol at the same stirring speed.

A similar operational stability study was made in the pilot-scale STR with continuous addition of 1.5 eq 96% ethanol over 2 h, at a P/V of 0.5 W/L. As Figure 6 shows, there is a little activity loss through four repeated uses in terms of both final FAEE yields after 6 hours and initial rates without any washing to the catalyst between batches. It indicates that the scaled STR at studied conditions can provide a similar environment as that presented in the lab-scale STR to maintain the stability of the lipase. The degradation of activity does seem marginally faster in the pilot scale, which could be related to the catalysts being stored with fresh oil between batches.

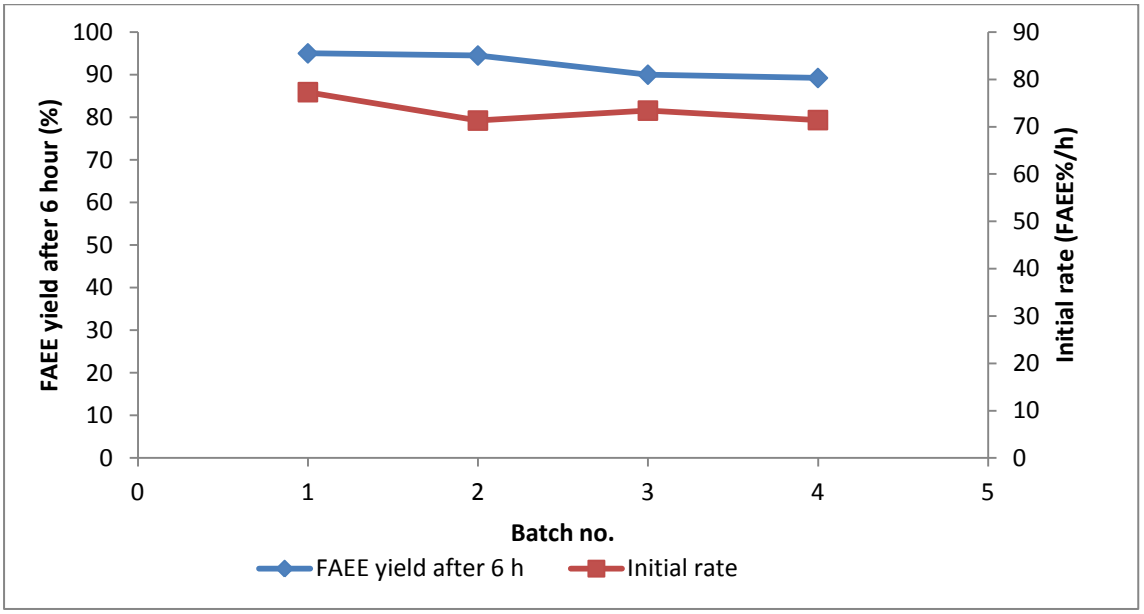

Figure 6 Operational stability of NS 88001 in pilot-scale STR (0.5 W/L)

3.6 Mechanical stability of NS 88001

In addition to the ability of retaining its synthetic activity through reuse, a good mechanical stability of the carrier of the immobilized lipase is also highly desirable for the reproducibility of the process. For a given carrier of immobilized enzyme, the damage that catalyst particles experience in an agitated tank varies with many factors, such as catalyst loading, power input, impeller type and the resulting physical properties of fluid.

The experiments investigating mechanical stability of NS 88001 in both lab- and pilot-scales were performed with catalyst particles pre-sieved to a size larger than 500 μm. The fractions of particles above 500 μm after being stirred at different conditions for 120 hour were measured and results are shown in Table 3 and Figure 7.

Table 3 Mechanical stability of NS 88001 in lab-scale STR with different mixtures

	Reaction mixture	Biodiesel		Mixture of glycerol and rapeseed oil			
Catalyst loading	5%	5%		5%		2.5%	
Viscosity[a]	Changeable (4-29)	7		85*		78*	
Re	450-5000	1800-3000		150-230		170-250	
Stirring speed[b]	Fraction dp>500μm	P/V[c]	Fraction dp>500μm	P/V[c]	Fraction dp>500μm	P/V[c]	Fraction dp>500μm
800	92	0.50	98	0.58	94	0.59	97
1000	81	0.98	88	1.14	85	1.15	87
1200	78	1.69	87	1.98	84	1.98	86

*rough estimations by treating glycerol droplets under the stirred condition as solids applying equation A.4 due to a high viscosity ratio of glycerol and rapeseed oil (Paul et al., 2003)
[a] unit: cP; [b] unit: rpm;[c] unit: W/L

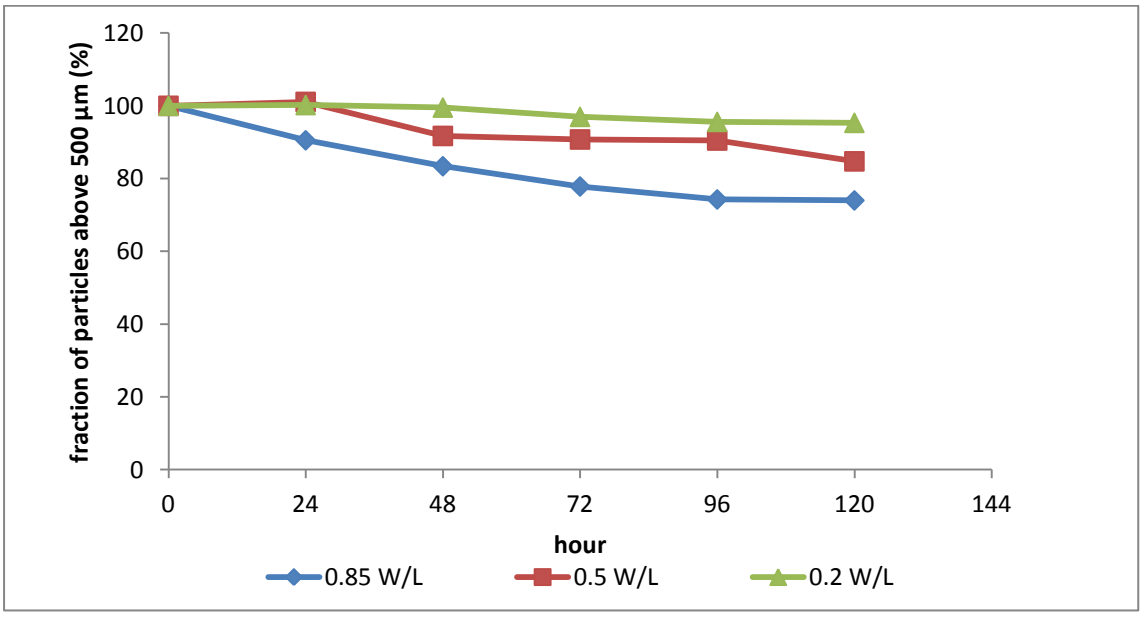

Figure 7 Mechanical stability of NS 88001 tested in pilot-scale STR

3.6.1 Effect of P/V and stirring time

In general, the shear damage to the catalyst particles increased with increasing P/V, correlated to the increasing shear stress applied on particles, as can be seen in Table 3 and also in Figure 7. Results in lab and pilot correlate well, indicating that 81% (lab scale) and 74% (pilot scale) particles were still larger than 500 μm after 120 h mixing at a P/V close to an industrial application (1.0 W/L). Stability was improved by reducing the power input in the pilot plant; 95% was obtained at a minimum P/V (0.2 W/L). Figure 7 also shows the particles slowly degraded in size as stirring time increases at any tested P/V.

3.6.2 Effect of catalyst loading

NS 88001 particles at a higher catalyst loading (5%) were subject to slightly more severe mechanical damage than a lower catalyst loading (2.5%) as shown in the results from the simulated mixture of oil and glycerol (Table 3), which can be explained by more collision of particles occurred at a higher density of catalyst.

3.6.3 Effect of viscosity

The results also show that the mechanical stability of NS 88001 particles did not have a significant difference in the biodiesel and in the mixture of glycerol and oil, a little worse in the latter. Although the viscosity in the latter mixture is much higher than the former mixture, the flow patterns in both mixtures are transitional regime (Table 3). It indicates that in the transitional regime the fluid viscosity did not show a significant effect on the shear stress imposed on the particles by the impeller.

3.6.4 Effect of reaction mixture

Compared to the other mixtures, NS 88001 particles were less mechanically stable in the reaction mixture, as shown in Table 3. While being repeatedly used in the reaction in the lab-scale STR, the viscosity and bulk density of the reaction mixture vary along with the reaction, which causes Re changes over a large range (450-5000) and results in varied turbulence intensities.

The physical properties of the reaction mixture containing about 95% biodiesel and 0.5 eq ethanol in the pilot-scale test are relatively constant because there was no repeated reaction involved. However, the flow pattern changed from transitional regime to turbulent flow due

to the greatly increased Re (>15000) as a consequence of using the constant P/V for scaling-up. The increased turbulence can cause more shear damage to the particles, which can probably explain the somewhat lower mechanical stability of NS 88001 in the pilot-scale STR than in the lab-scale STR, 74% and 85% of particles remaining larger than 500 μm at 0.85 and 0.5 W/L in pilot scale (Figure 7), compared to 81% and 92% at similar P/Vs in lab scale (Table 3). The other consequence of this scale-up approach is the increased tip speed, which can also explain the increased mechanical stress from the impeller. Additionally, the carrier material of NS 88001 is probably not stable with the reactant ethanol, which makes the carrier more vulnerable to the shear stress in the reaction mixture.

However, no biocatalyst particles smaller than 300 μm were found in any of the 120 hours samples in the pilot-scale test, mining that there was only a very limited risk of clogging of or passing through the filter (mesh size 36 μm), installed near the bottom of the tank for retaining the biocatalyst. This is promising for the industrial implementation.

4. Operating window

An operating window (Woodley and Tichener-Hooker, 1996) is given in Figure 8 to summarize some of the findings from this study of immobilized lipase-catalyzed transesterification and its scale-up. It is defined by two variables, which are P/V and catalyst loading and their relations with other parameters.

A sufficient catalyst distribution in STR is required to an efficient use of catalyst, which was found to be above a P/V of 0.2 W/L corresponding to Njs. This sets a vertical boundary in the graph. This boundary can be moved towards higher P/V region due to the glycerol effect, at least 0.5 W/L in the case of using absolute ethanol. However, less agitation power is required to remove this effect when using 96% ethanol, which is possibly between 0.2 and 0.5 W/L according to the obtained results.

Significant mass transfer limitation was observed at a high catalyst loading of 10%. For a reasonable space-time-yield of STR, the catalyst loading is set to be above 2.5%. Two horizontal boundaries are subsequently added to the graph.

The combined effect of P/V and catalyst loading on the mechanical stability of NS 88001 in STR is also shown in Figure 8. In the pilot-scale STR, 85% of the catalyst particles remained above 500 μm with a 5 wt% catalyst loading at 0.5 W/L after 120 h. Assuming this result is

set as a constraint, a boundary based on an allowance of 15% mechanical damage to catalyst is added to the graph.

The resulting operational window can guide the operations of NS 88001 catalyzed transesterification in STR to achieve the maximum process efficiency.

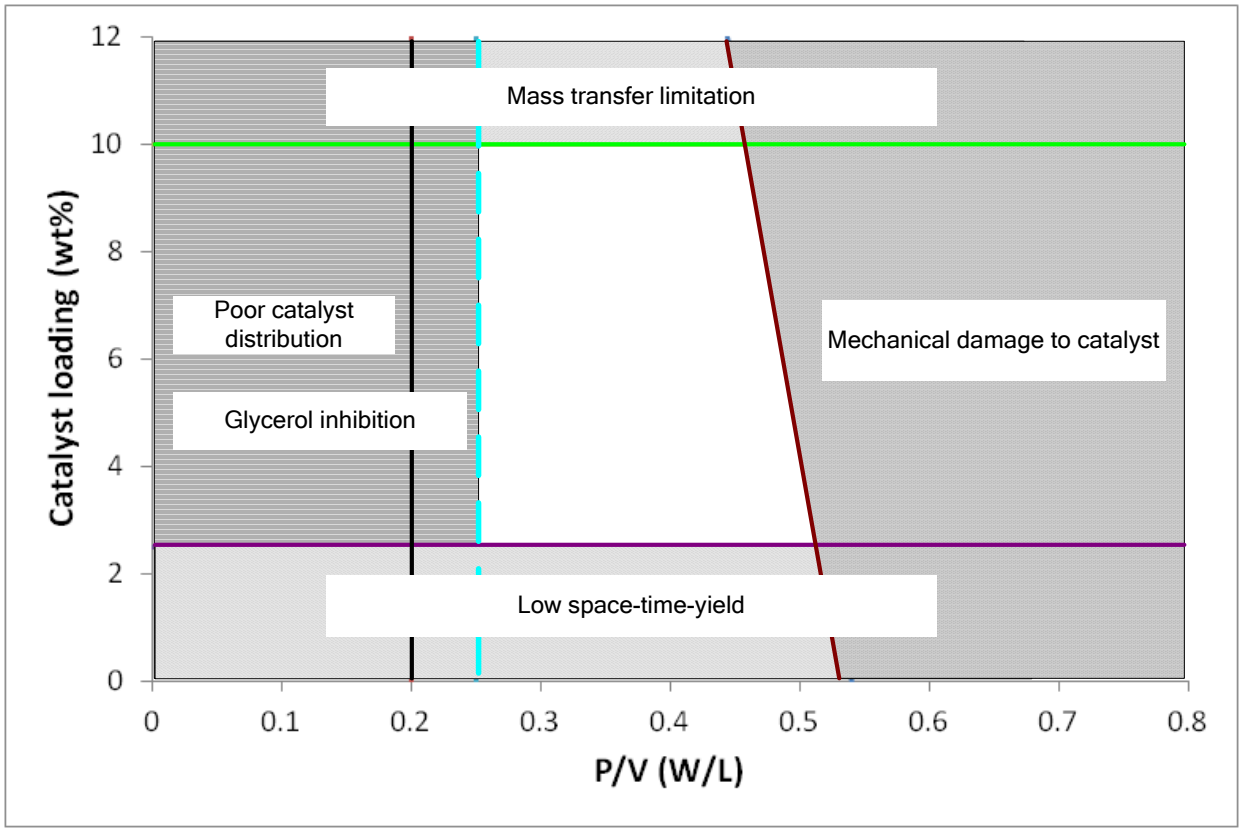

Figure 8 Operating window for STR (the dash line is related to the power input for removing glycerol when using 96% ethanol)

5. Conclusions

To achieve an efficient NS 88001-catalyzed process, the minimum power input ($P/V_{minimum}$) should be based on Njs. Any P/V higher than the $P/V_{minimum}$ did not show significant improvement on the catalyst performance in both lab- and pilot-scale STRs.

The lab-scale results can almost be reproduced in pilot-scale STR with respect to initial rate, reaction progress and operation stability of NS 88001, which means the scaling-up approach based on constant P/V is successful. Mechanical stability of NS 88001 in pilot-scale STR is a little worse than that in lab-scale STR. No fine particles were found falling into the risky zone of clogging or pass through the filter.

Absolute ethanol is not preferable to the operation stability of NS 88001 in the studied reaction system because it requires more cautions in operations. As a contrast, using 96% ethanol can improve the stability of NS 88001 and make it more adaptable to different conditions in STR.

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Appendix

A.1 Viscosity of liquid blend

The liquid phase of the studied reaction mixture can involve ethanol, biodiesel, glycerides and glycerol when it is produced as the byproduct. Refutas method has been employed to estimate the viscosity of a liquid mixture and the equations are given as follows (Maples, 2000):

$$VBI = 10.975 + 14.535\, ln[ln(v + 0.8)] \qquad A.1$$

$$VBI_{blend} = x_1 VBI_1 + x_2 VBI_2 + \ldots + x_N VBI_N \qquad A.2$$

$$v_{blend} = exp\left[exp\left(\frac{VBI_{blend} - 10.975}{14.535}\right)\right] - 0.8 \qquad A.3$$

Where:

VBI is the viscosity blending index

VBI_i is the viscosity blending index of each component in the liquid mixture

VBI_{blend} is the viscosity blending index of the liquid mixture

X_i is the mass fraction of each component in the liquid mixture

v is the kinematic viscosity in centistokes

A.2 Viscosity of solid and liquid mixture

The viscosity of the immobilized lipase-catalyzed reaction mixture is estimated according to Vand equation for the mixture of liquid and spherical particles, whose volumetric concentration is higher than 2% (Vand, 1948).

$$\mu_c = \mu e^{2.5C/(1-0.609C)} \qquad A.4$$

Where μ_c is the viscosity of the solid and liquid mixture, μ is the viscosity of the fluid, C is the volume fraction of the solid phase, e is the natural exponential.

A.3 Just suspended speed (Njs)

The just suspension speed is defined as the minimum agitation speed for lifting all particles off the bottoms. Normally it can be a waste of energy input if the impeller speeds are set far above Njs. Therefore, Njs is a practical parameter for guiding the design of reactors and impellers for solid-liquid suspension. The empricial estimation of Njs is given by equation:

$$Njs = Sv^{0.1}\left[\frac{g_c\,(\rho_s - \rho_l)}{\rho_l}\right]^{0.45} X^{0.13} d_P^{0.2} D^{-0.85} \qquad A.5$$

D is the impeller diameter (m); d_P is the mass-mean particle diameter (m), X is the mass ratio of suspended solids to liquid *100 (kg solid/kg liquid); S is the dimensionless number which is a function of impeller type, as well as of D/T and C/T and in the studied reactors S is approx. 5.2; v is the kinematic viscosity of the liquid (m^2/s); g_c is the gravitational acceleration constant, 9.81 m/s^2; ρ_s and ρ_l are the densities of particle and the density of liquid (kg/m^3).

A.4 Power input (P)

For the purpose of scale up, it is useful to know the power consumption in this stirred tank reactor. The power input can be calculated with the following equation:

$$P = N_p \rho N^3 D^5 \qquad A.6$$

Where N_p is the power number which is dependent on impeller type and impeller Reynolds number, ρ is the bulk density, N is the rotation speed and D is the diameter of the impeller. The impeller Reynolds number is given by

$$Re_{imp} = \frac{\rho N D^2}{\mu} \qquad A.7$$

where μ is the viscosity of the bulk liquid.

The flow regime is laminar flow for Re below 10; transition region is between 10 and 10000 and turbulent above 10000.

PAPER 3

A two-stage enzymatic ethanol-based biodiesel production in a packed bed reactor

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A two-stage enzymatic ethanol-based biodiesel production in a packed bed reactor

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Abstract

A two-stage enzymatic process for producing fatty acid ethyl ester (FAEE) in a packed bed reactor is reported. The process uses an experimental immobilized lipase (NS 88001) and Novozym 435 to catalyze transesterification (first stage) and esterification (second stage), respectively. Both stages were conducted in a simulated series of reactors by repeatedly passing the reaction mixture through a single reactor, with separation of the by-product glycerol and water between passes in the first and second stages, respectively. The second stage brought the major components of biodiesel to 'in-spec' levels according to the European biodiesel specifications for methanol-based biodiesel. The highest overall productivity achieved in the first stage was 2.52 kg FAEE(kg catalyst)$^{-1}$h^{-1} at a superficial velocity of 7.6 cm min^{-1}, close to the efficiency of a stirred tank reactor under similar conditions. The overall productivity of the proposed two-stage process was 1.56 kg FAEE(kg catalyst)$^{-1}$h^{-1}. Based on this process model, the challenges of scale-up have been addressed and potential continuous process options have been proposed.

Keywords: biodiesel, lipase, packed bed reactor, scale-up

1. Introduction

Biodiesel provides one of the possible sustainable solutions to the depletion of fossil fuels (Jegannathan et al., 2008). Fatty acid methyl ester (FAME) is the conventional form of biodiesel derived from vegetable oil, typically soybean oil or rapeseed oil, and methanol via alkali-catalyzed reactions (Akoh et al., 2007). The product recovery process usually includes the neutralization and removal of the soluble catalyst (Zhang et al., 2003). An alternative process that is gaining more and more interest uses lipase as a catalyst and ethanol (rather than methanol) as the acyl acceptor, resulting in biodiesel in the form of fatty acid ethyl ester (FAEE). While FAEE-biodiesel is not yet recognized as biodiesel, it comes with several advantages. Use of ethanol reduces the dependence on a fossil-fuel derived alcohol substrate (Al-Zuhair, 2007). Additionally, ethanol is actually preferred by lipase (Kumari et al., 2007). The lipase-catalyzed reactions have the advantages of low energy consumption, reduced formation of by-products and waste (Nielsen et al., 2008).

A biodiesel production process based on immobilized lipase-catalysis is a multi-phasic system throughout the reaction, including the insoluble phase of the biocatalyst, lipid phase and additionally a polar phase when alcohol exceeds the solubility limit (or when the by-product glycerol is present). Stirred tank reactors (STRs) and packed bed reactors (PBRs) are often used as reactors for studying this type of reaction at different scales.

Biodiesel production at a large scale is commonly accommodated in batch STRs because of the ease of construction, operation and maintenance. The disadvantage of the STR is that it can introduce mechanical damage to the biocatalyst, which can reduce the reusability of the catalyst and affect the economic feasibility of the processes (Halim et al., 2009). In addition, downtime cannot be avoided in batch STR operation, which leads to reduced total space-time yield (Balcáo et al., 1996). A PBR reduces the level of shear stress to catalyst particles and enables continuous operation, (Balcáo et al., 1996; Halim et al., 2009). However, the PBR can be limited by a high pressure drop over the bed or obstruction of the catalyst bed by accumulation of insoluble components from the reaction mixture.

Due to the multi-phasic reaction system most studies on immobilized lipase-catalyzed biodiesel production in a PBR involve the use of a solvent to reduce the viscosity of the reaction mixture, and enhance the solubility of alcohol in the feedstock as well as dissolve the by-product glycerol, improving the mass transfer and allowing operation within a single liquid phase.

The most often used co-solvents in immobilized-lipase catalyzed transesterification in PBRs are n-hexane and tert-butanol (Dossat et al., 1999; Royon et al., 2007; Shaw et al., 2008). Both co-solvents have shown some positive effects on increasing the reaction rate and yield. However, many co-solvents are volatile and flammable, difficult to handle in large amounts and they also pose an environmental hazard. The co-solvent must also be recovered, which will add to the cost of production and make scale-up more complicated. From this perspective an efficient solvent-free PBR system can be more promising.

To solve the deactivation of lipase by the un-dissolved alcohol, researchers have developed a step-wise reaction scheme for a PBR where alcohol is added in portions and in this way a good stability of the immobilized lipase can be obtained (Watanabe et al., 2000). In addition, a further problem that needs to be addressed to achieve an efficient transesterification in a PBR with an immobilized lipase is that the catalyst can be clogged by accumulation of the glycerol by-product inside the reactor and thus inhibit the enzyme (Belafi-Bako et al., 2002; Xu et al., 2011). Hama and co-workers found that the flow velocity was important to the removal of glycerol from a PBR but that the reaction progress was not noticeably affected by the flow velocities they studied (Hama et al., 2011).

Most studies about biodiesel production focus on reaction rate and biodiesel yield in a single transesterification stage. However, few studies have been published on making biodiesel meet product specifications (Hama et al., 2011). The commonly-used biodiesel specifications in USA and Europe (ASTM D6751 and EN 14214), both show very low tolerance for impurities in biodiesel (Knothe, 2006). Meeting these specifications in a single reaction stage, without separation of glycerol and further processing (through conversion or separation), is exceptionally difficult. Therefore, in this work the conversion of glycerides to biodiesel run into two stages, with separation of glycerol in-between. This also allows the use of two different biocatalysts to improve the efficiency of the process. The first stage is primarily a transesterification reaction carried out by the immobilized *Thermomyces lanuginosus* lipase (NS 88001), and converts triglyceride (TAG) using 96% ethanol, which is advantageous from an economical point of view. The major reaction of the second stage is esterification with dry ethanol, catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435). Both stages have been studied in a solvent-free PBR system with respect to the effects of flow velocity as well as ethanol and catalyst loading. The efficiency of PBR in this work has also been compared to an STR system and other reported studies on PBRs.

In addition an operating window for a PBR has been provided to define and visualize the feasible operating parameters. The potential and challenges in scaling up the process are also addressed based on the experimental results obtained in this study.

2. Theory

2.1 Residence time

As a plug flow reactor, conversion in a PBR is a function of axial position along the reactor, assuming that all of the substrates pass through the whole cross-sectional area of the PBR at the same velocity, with an identical residence time under ideal conditions. The residence time (t) correlates to the position within PBR, superficial velocity and the bed porosity, as given by equation 1.

$$t = \frac{L}{v_s}\varepsilon \quad (1)$$

Where L is the length of the catalyst bed, v_s is the superficial velocity of the fluid (volumetric flow rate divided by the cross-sectional area of the bed), and ε is the void fraction of the bed.

2.2 Reynolds number for porous media in a packed bed reactor

The Reynolds number (Re) for flow through a packed bed of identical and spherical porous particles is defined by equation 2.

$$Re = \frac{\rho v_s d_s}{\mu(1-\varepsilon)} \quad (2)$$

Where ρ is the fluid density, μ is the fluid viscosity, and d_s is the diameter of the spherical particles.

For Re < 10 the fluid is in the laminar flow regime, Re > 2000 implies turbulent flow.

2.3 Pressure drop

According to Darcy's law, which is an empirical observation, the pressure drop for laminar flow through a column packed with porous medium can be expressed by equation 3.

$$\Delta P = \frac{\mu L v_s}{\beta} \quad (3)$$

Where ΔP is the pressure drop, L is the length of the column, β is the permeability of the porous medium ($= \frac{\varepsilon^3 d_S^2}{180(1-\varepsilon)^2}$), which is related to the porosity of the bed and the size of the packing particles (Bird et al., 2002).

3. Materials and methods

3.1 Materials

Rapeseed oil was kindly donated by Emmelev A/S (Otterup, Denmark). Absolute ethanol (≥ 99.9%) was purchased from Fluka Chemie (Buchs, Switzerland). n-Heptane (≥99%) and t-butyl methyl ether (≥ 99.8%) were purchased from Sigma–Aldrich (Steinheim, Germany) as HPLC grade. HPLC standards (≥99%), specifically oleic acid, ethyl oleate, 1,3-diolein and 2-monoolein, were all purchased form Sigma-Aldrich (Steinheim, Germany).

Three immobilized lipase catalysts were used in the study: Novozym 435 (N435) is *Candida antarctica* lipase B (CALB) immobilized on a macroporous divinylbenzene-crosslinked polymethylmethacrylate PMMA); TL IM is *Thermomyces lanuginosus* lipase (TLL) immobilized on a silica carrier; NS 88001 is an experimental catalyst that consists of TLL immobilized on a hydrophobic polymeric resin. All of the biocatalysts used in this study were kindly donated by Novozymes A/S (Bagsværd, Denmark).

3.2 Reactor set-up and reactions

STR

The reactor is a glass tank implemented with a 4-blade marine propeller driven by a motor and baffled with four flat blades. The reactor is submerged in a water bath to maintain the temperature required for the reactions.

Transesterification of rapeseed oil in STR

The reaction mixture was composed of 100 g rapeseed oil, 5 % catalyst (NS 88001) and a total of 27 mL 96% ethanol, corresponding to 1.5 molar equivalents (eq) of the total fatty acids in the oil. The ethanol was added in three equal portions (0.5 eq) at 0, 2 and 4 hours reaction time. Reactions were performed at 35 ^{o}C. Samples (50 µL) were taken from the tank at regular intervals for HPLC analysis.

PBR

The reactor is a glass column (25 cm*1 cm) from Omnifit® (Cambridge, UK) with a water jacket connected to a circulating water bath to maintain the temperature. Frits are used in both

ends of the column to retain catalyst particles. A pressure sensor was placed at the inlet of the column and data was collected by Logger Lite 1.2 (Vernier Software and Technology, Beaverton, OR, USA).

Transesterification of rapeseed oil in PBR

3.4 - 5.4 g catalyst (NS 88001) was packed in a column, resulting in a bed length of approximately 16 - 22 cm. Transesterification was carried out in three steps. In first step a mixture of 108 g rapeseed oil and 0.25-0.5 eq 96% ethanol was pumped into the reactor in a down-flow manner via a peristaltic pump at a flow rate of 0.14 - 6 mL min^{-1}. After all the reaction mixture passed through the column, any glycerol by-product that separated into a second phase was allowed to settle before the oil phase was pumped through the column again. This procedure was repeated until equilibrium conversion was reached. Then another 0.5 eq 96% ethanol was added into the reaction mixture on the second and the third step.

Esterification in PBR

5.4 g N435 or NS 88001 was packed into a column of about 22 cm long. The esterification reaction was carried out in multiple steps of a single pass through the column.. For the first (esterification) step the substrate obtained from first stage (transesterification) was dried in a vacuum oven (90 ^{o}C, 200 mbar) overnight and then mixed with 5 v/v % (approx. 0.3 eq) absolute ethanol before use. The substrate was supplied by a peristaltic pump in down-flow mode through the column in a single pass at a fixed flow rate of 1.0 mL min^{-1}. The drying procedure was repeated with the eluate before mixing with another portion of 5 v/v % dry ethanol for the next step.

3.3 HPLC analysis of reaction mixtures

Samples (55 μL) were dissolved and diluted in a mixture of 0.5 mL acetic acid and n-heptane (4:1000) and further diluted 100-fold in the same mixture to achieve concentrations of around 1.0 mg mL^{-1}. 40 μL of the solution was injected on an HPLC (Dionex A/S, Hvidovre, Denmark) for analysis of the composition of FAEE, FFA, TAG, DAG and MAG. The HPLC was equipped with a U3000 autosampler, TCC-3000SD column oven and U3400A quaternary pump modules. A Corona® Charged Aerosol Detector (Thermo Scientific Dionex, Chelmsford, MA, USA) was used for detection with a nitrogen flow of a pressure 35.0 psi. The separation was done on a 250 mm × 4.0 mm cyanopropyl column (Discovery® Cyano) from Sigma–Aldrich (Steinheim, Germany) at a flowrate of 0.75 mL min^{-1}. Program

control, data acquisition, and analysis were carried out using Chromeleon 6.8 software. A binary gradient program was applied using phase A: 99.6% n-heptane, 0.4% acetic acid; and phase B: 99.6% t-butyl methyl ether, 0.4% acetic acid (Foglia, 1997). An example chromatogram is shown in figure 1, with all sample components of interest indicated. The method was calibrated using ethyl oleate and rapeseed oil feedstock (0 to 40 µg injected on column), 1,3-diolein and 2-monoolein (0 to 25 µg injected) and oleic acid (0 to 8 µg injected). Within the indicated range, injected mass could be described as a function of peak area by a second-order polynomial with a correlation coefficient over 0.995 for all components.

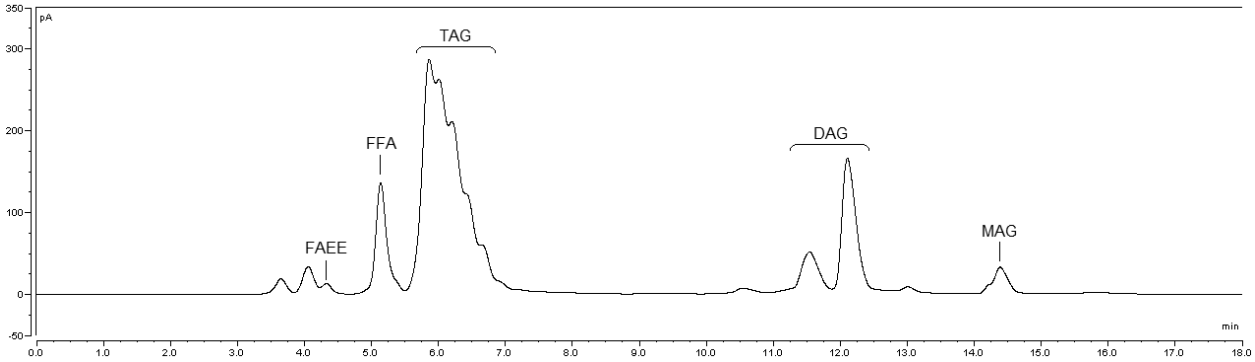

Figure 1 Example chromatogram for HPLC method, showing peaks for FAEE, FFA, TAG, DAG and MAG from a transesterification reaction with rapeseed oil.

4. Results and discussions

4.1 First stage reaction (transesterification)

4.1.1 Effect of ethanol amount

The solubility of 96% ethanol in rapeseed oil at 35 ^{o}C is about 0.5 eq (preliminary experimental result), under which conditions the lipase catalyzes the reaction in a single homogenous phase. To investigate the effect of the ethanol amount within the solubility limitation on the reaction, a single pass through a column packed with NS 88001 (bed length 16 cm, superficial velocity 1 cm min^{-1}) was carried out for ethanol loads of 0.25 and 0.5 eq. The FAEE yield was more than two-fold higher at 0.5 eq ethanol loading than 0.25 eq loading (7.9 vs. 3.2 %, respectively), which can be explained by the increased (but still non-inhibitory concentration of substrate) and better mass transfer for substrate in a less viscous mixture with a higher ethanol loading. Therefore, 0.5 eq ethanol was chosen for all following experiments.

4.1.2 Effect of enzyme loading

Lipozyme TL IM and NS 88001 are the same TLL immobilized on different carriers. Their performances in a PBR for transesterification have been compared here by looking at the effect of their loadings, which was studied by varying the length of catalyst bed in a PBR at the same flowrate.

For a given flowrate varying the length of catalyst bed can change the residence time. Therefore, a higher conversion can be expected from a longer catalyst bed. As the results shown in Table 1, the conversions in 22cm-long beds of both TL IM and NS 88001 were higher than 16 cm-long beds for both steps. The overall reaction rates in both lengths of catalyst bed were expected to be either equal or decreasing as a result of increasing the residence time. However, for each catalyst the reaction rates in the longer bed length were found to be higher than those in the shorter length for both steps. In other words, the reaction was much faster in the extra length of the bed. This can possibly be explained by a decrease in viscosity of the reaction mixture along with the bed as some FAEE and partial glycerides are produced in the first part of the bed. This could allow better mass transfer of substrates in the reaction in the last part of the bed. This gives the conclusion that there is no disadvantage of using a full length of the column as the catalyst bed and comes with the added benefit of minimizing the number of passes through the bed in the next experiments.

Table 1 Results of the effect of enzyme loading on transesterification in PBR

Catalyst	Catalyst bed length[a]	Residence time[b]	After 1st step		After 2nd step	
			FAEE yield[c]	Reaction rate[d]	FAEE yield[c]	Reaction rate[d]
TL IM	16	11.2	7.1	0.63	11.2	0.37
TL IM	22	15.4	13.3	0.86	19.8	0.42
NS 88001	16	11.2	7.9	0.71	19.3	1.02
NS 88001	22	15.4	15.7	1.02	35.5	1.29

Reaction conditions: 0.5 eq 96% ethanol was added before step 1 and another 0.5 eq before step 2. Superficial velocity was 1 cm min^{-1} for all experiments.
Each step consists of a single pass through the column, with the rate based on the conversion carried out in that stage.
[a.] unit: cm; [b.] unit: min; [c.] mass %; [d.] unit: FAEE% min^{-1}

TL IM and NS 88001 had close conversions in the first step with the same bed length, which is because they have similar lipase loadings on their carriers. However, with another 0.5 eq ethanol added at the second step, the reaction rates of TL IM were reduced and conversions of the second step by TL IM were also lower compared to those by NS 88001, which were as expected accelerated by increased ethanol concentrations in the second step. We have postulated that this is related to the different interactions of by-product glycerol and the different carriers of the two catalyst types. As more glycerol was formed in the second step than the first step (observed by dyeing glycerol *in-situ*, not shown), more particles of TL IM were likely clogged by glycerol, which has a stronger affinity for the hydrophilic carrier of TL IM than for the hydrophobic carrier of NS 88001. The glycerol layer formed on the outside of the catalyst particles can cause serious mass transfer limitation for the hydrophobic substrate to approach the lipase (Xu, et al., 2011). Based on these results NS 88001 was selected to carry out all following experiments.

4.1.3 Effect of flow velocity on activity

Figure 2 shows how the FAEE yield varies as function of the superficial velocity (the corresponding Reynolds numbers based on the starting reaction condition are also included in the figure). Clearly, the residence time is one of the major factors affecting the conversion. However, too low a velocity may create a thicker stagnant layer for substrate diffusion. Therefore, an optimal velocity with respect to conversion in a single pass was investigated, which was found to be around 1 cm min^{-1}. Interestingly, the reaction rate, here defined as mass-% of FAEE formed per residence time, increases almost linearly with the increasing velocity (Figure 2). This can be explained the mass transfer is improved at high flow velocities even though the Reynolds number indicates laminar flow at all tested velocities. The pressure drop is 1.1 bar at 7.6 cm $min^{-1}$and therefore higher flow rates were not tested due to limitations in the present set up This is also the reason why high velocities were used in the following experiments since the limited residence time could be overcome by increasing the number of passes of the reaction mixture through the column.

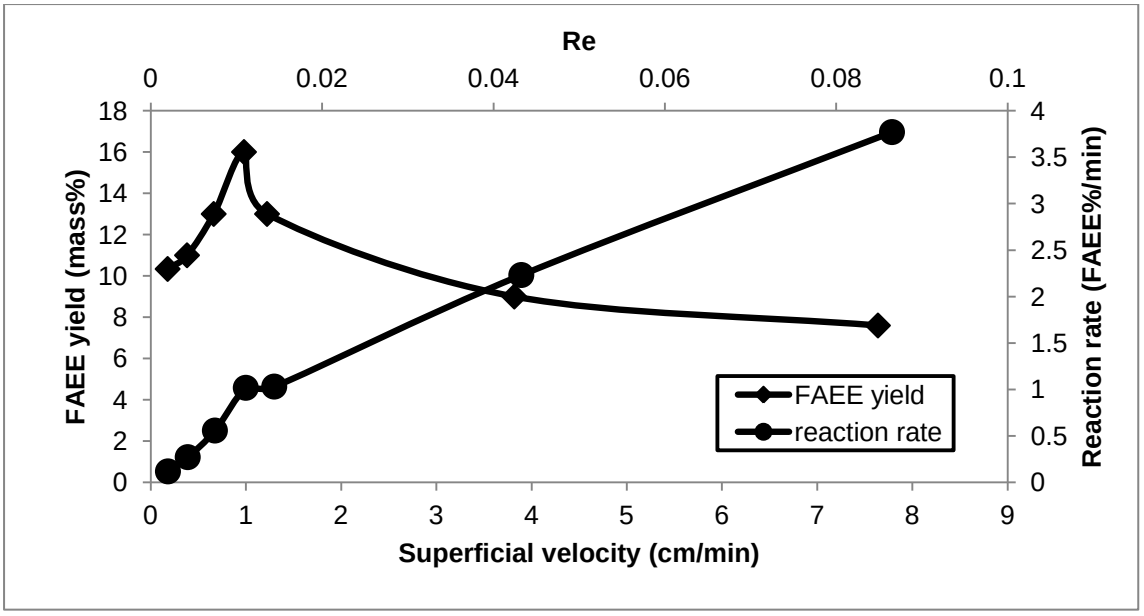

Figure 2 Effect of flow velocity on FAEE yield and reaction rate (defined as FAEE yield per residence time). FAEE yield and reaction rate are based on a single pass through the catalyst bed (22 cm) with 0.5 eq 96% ethanol

The reaction time-courses of transesterification in a simulated series of PBRs at various velocities were compared with the performance of the same catalyst and loading relative to the oil substrate in an STR (Figure 3). The required reaction time decreased steadily as the velocity increased and finally overlapped with that in an STR at 7.6 cm min^{-1}. The flow patterns in the two reactor types under the compared conditions are still different; laminar flow in PBR (Re: 0.09-0.35) and transitional flow in STR (Re: 700-2500) through the whole reaction. The effect of flow velocity on the reaction rate was more obvious in the first step because the reaction curve turns to be much steeper as the velocity increased in the first step whereas reaction curves are almost parallel at the second and third step, as can be seen in Figure 3. It is very likely that the reaction rate is limited by external mass transfer to the greatest extent in this first step where the reaction mixture is relatively more viscous, and the potential reaction rate the highest. Thus, higher reaction rate was observed with higher flow velocity which could improve the mass transfer. At higher conversion, both the viscosity and potential enzymatic activity are reduced but the reaction rates are higher than in the first step and closer between different velocities, indicating that mass transfer is less limiting in the latter steps. It also indicates that the by-product glycerol did not have a significant effect on the mass transfer at the tested velocities even though it was observed that the accumulation of glycerol in the column varied with the flow velocity (a dye was used to visualize glycerol; photos not shown) a faster flow resulted in less accumulation. This is consistent with

previous findings by Hama and coworkers, who found that although a higher flow velocity can remove more glycerol from the column, this does not greatly affect the reaction progress (Hama et al., 2011).

A yield of about 93% FAEE yield was achieved in a PBR, which was slightly lower than that in a STR (95%). This can probably be explained by some of the ethanol lost in the glycerol which was removed between passes.

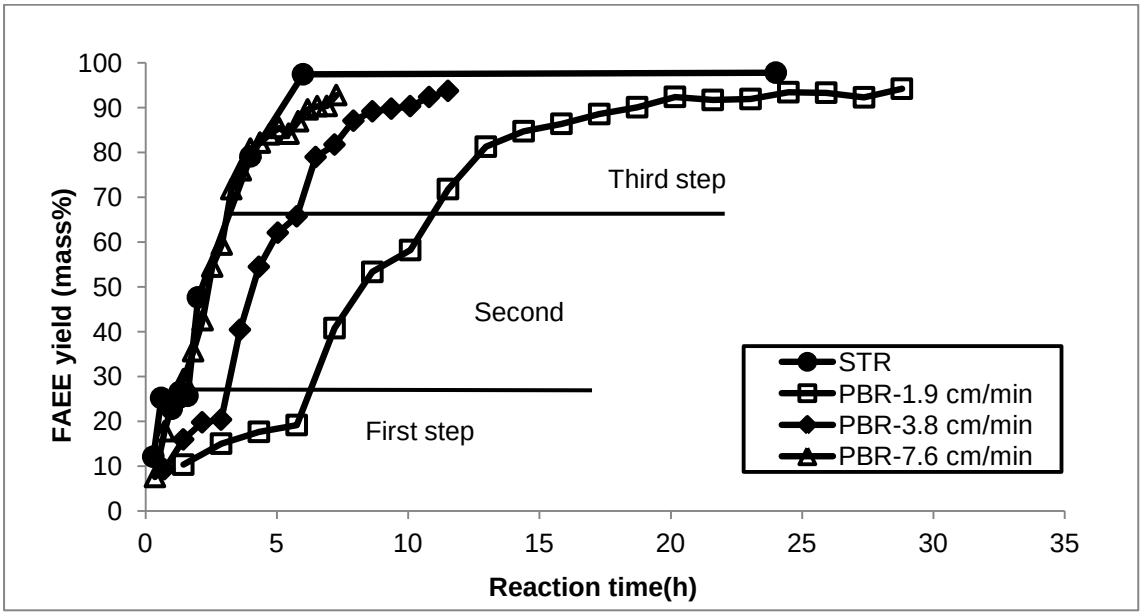

Figure 3 Comparison of transesterification in STR and PBR with different flow velocity. Each data point indicates the FAEE conversion of each pass through the catalyst bed (22 cm) with 0.5 eq 96% ethanol added at each step

A common motivation for many studies on PBR in enzymatic biodiesel production is that it can offer an opportunity for continuous biodiesel production. The experiments in this study were conducted by repeatedly passing the reaction mixture through a single column to simulate the effects of continuous production in a series of columns. In this work we obtained 92.8% FAEE after the reaction mixture experienced 20 passes through the column at the flow velocity 7.6 cm min^{-1}. This corresponds to a productivity of FAEE of 0.042 g FAEE(g catalyst)$^{-1}$ min^{-1}. In the same manner the productivities from other published work on PBRs has been calculated and compared to the productivities of NS 88001 in batch STR and continuous PBR in Table 2. The productivity of NS 88001, presented here is higher than that achieved by Hama and coworkers using N435 and methanol, which shows that NS 88001 has a higher specific activity for ethanolysis than N435 for methanolysis under the studied conditions. The efficiency of the PBR system in this work is close to that of batch STR

system under similar conditions. This does not take into account the downtime that has to be considered when operating an STR in batch mode. The use of co-solvent makes the PBR system more efficient than the batch STR from this work with respect to productivity as reported by Royon and coworkers via one-pass through a column packed with N435, using methanol and t-butanol as the co-solvent (Royon et al., 2007). The flow velocity in their work was much lower than those in solvent-free PBRs, indicating that the co-solvent can improve mass transfer to a great extent. However, the disadvantages of using a co-solvent, limit the applications as explained previously.

Table 2 Productivity comparison

	FAEE Yield (%)	Flow velocity[a]	Catalyst	Productivity[b]	Alcohol	Total alcohol (eq)	Solvent
STR in this work	95		NS 88001	0.053[c]	EtOH	1.5	free
PBR in this work	92.8	7.6	NS 88001	0.042	EtOH	1.5	free
Hama et al., 2011	88.9	9.3	N435	0.023	MeOH	1.67	free
Royon et al., 2007	95	0.57	N435	0.067	MeOH	2	t-butanol

[a.] unit: cm min^{-1} ; [b.] unit: gFAEE (g enzyme)$^{-1}$ min^{-1}; [c.] reaction time 6 hours

4.2 Second stage reaction

The reasons for introducing a second stage are to make the product in-spec and also to further improve the yield of FAEE. To convert FFA into biodiesel, absolute ethanol is used in this stage.

A preliminary experimental result showed that the flow velocity had little effect on the reaction rate of N435 in this stage of reaction. Thus the superficial velocity was fixed at 1.27 cm min^{-1} (corresponding to 1.0 mL min^{-1}) to maintain a reasonable operation time and to achieve the equilibrium in a single pass through the column.

4.2.1 Effect of absolute ethanol loading

The effect of the amount of absolute ethanol in the esterification reaction was investigated, using the product from a transesterification of rapeseed oil and 1.0 eq 96 % ethanol. This

starting material contained approx. 95% FAEE and 3 % FFA. Since the substrate mixture, mainly composed of FAEE and partial glycerides, is readily miscible with ethanol, a range of ethanol loadings (5, 10 and 20 v/v %) were evaluated in this experiment.

The effect of ethanol loading on the behavior of the PBR until steady state is reached is shown in Figure 4. The tested ethanol loadings did not significantly affect the steady-state FFA content. It reached the steady state fastest with an ethanol loading of 10 v/v %. 20 v/v % ethanol did not reduce the FFA content as much as the other ethanol loadings over the single pass through the column, which could also be due to inhibition of the catalyst by the large excess of ethanol. Since there was no tangible advantage of using a higher loading of ethanol, 5 v/v % of absolute ethanol was chosen for the following experiments since it would be desirable to reduce the amount of ethanol to be recovered and recycled in an industrial application.

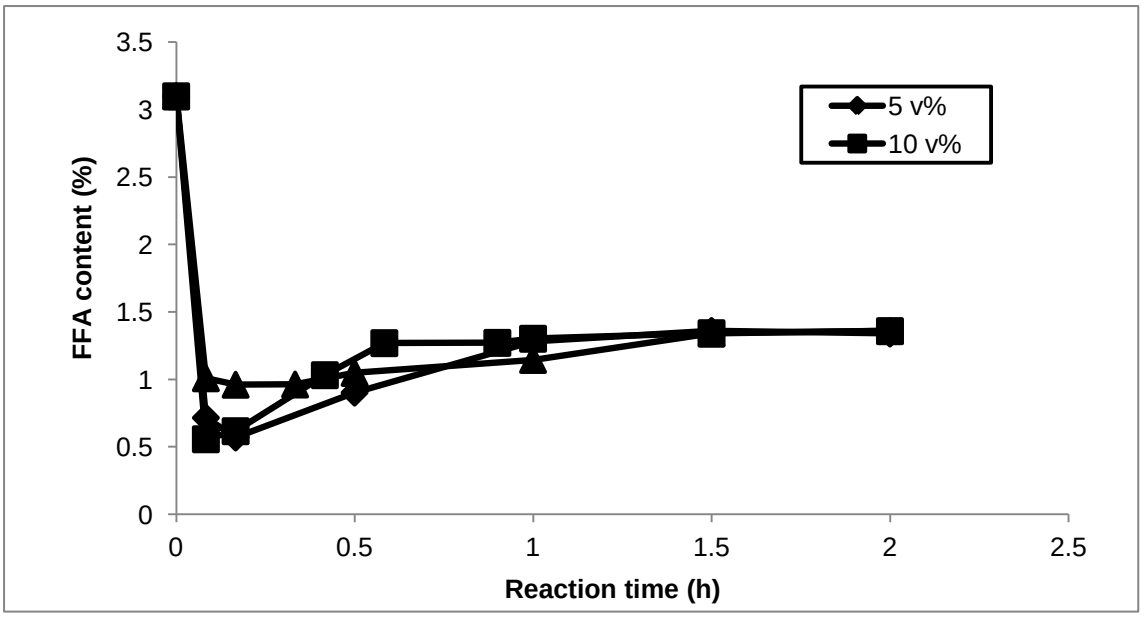

Figure 4 Effect of ethanol loading on esterification of FFA

4.2.2 Effect of catalyst type

The residual components in the product from the first stage are relatively small molecules such as FFA, MAG and DAG, together with a small amount of TAG. Previous studies indicate that TLL would be less useful for such substrates than less 1,3-positionally specific lipases such as CALB, the lipase used in N435 (Du et al., 2005), so an experiment was made to compare the performance of NS88001 to N435 in the second process stage. A transesterification product made using rapeseed oil and 1.5 eq 96% ethanol was used as the

starting material for this evaluation, with an initial FFA concentration of 1.5-1.7%. The results of repeated passes through columns packed with N435 (4 passes) and NS 88001 (5 passes) respectively are shown in Figure 5. The process using N435 requires 4 steps, consuming 20 v/v % ethanol in total, to successfully reduce the FFA content to 0.25 % (m/m), the upper limit for FFA content according to the EN 14214 biodiesel specification. Besides FFA content, the other four components (FAEE, TAG, DAG and MAG) after the 4-step reaction are actually also in spec (Table 3). The system using NS 88001, on the other hand, worked more slowly on FFA and did not achieve the required concentration even after 5 steps. The concentrations of products from the two catalysts listed in Table 3 show the use of NS 88001 resulted in lower levels of TAG and DAG than for N435, but also leaves more FFA and MAG in the product. This is consistent with the abovementioned difference in substrate specificities of the two lipases.

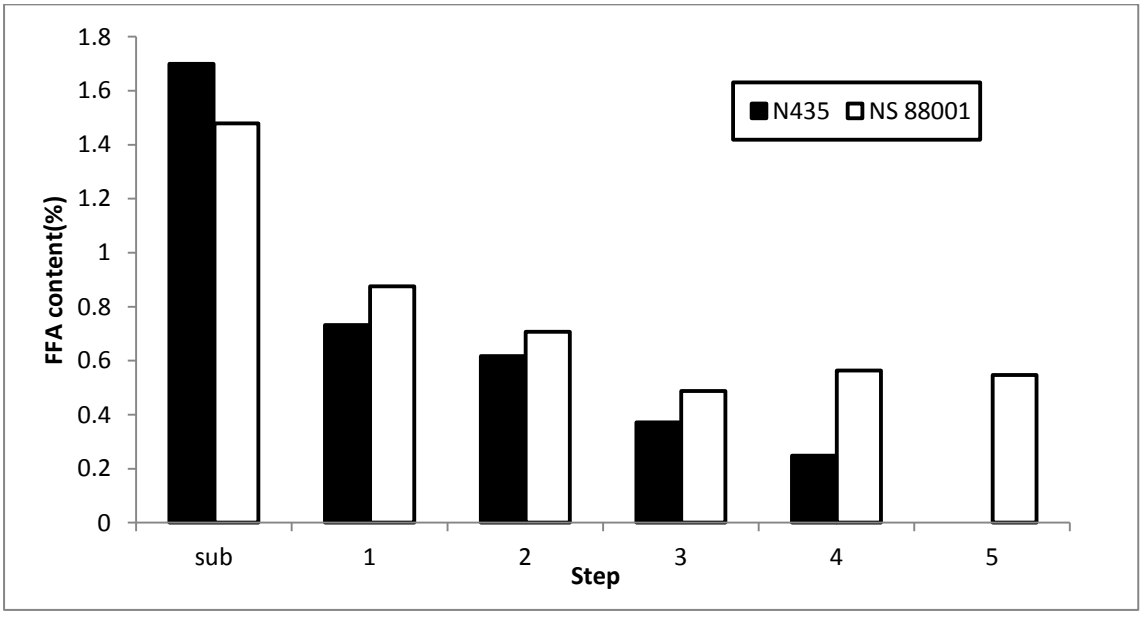

Figure 5 Results of stepwise esterification catalyzed by N435 and NS 88001. 4 reaction steps were carried out with N435 and 5 with NS 88001.

After this stage, the overall productivity decreased to 0.026 g FAEE(g catalyst)$^{-1}$min^{-1} which is however still comparable to 0.023 g FAME(g catalyst)$^{-1}$min^{-1} obtained by Hama and coworkers (Hama et al., 2011). The overall efficiency of the two-stage process can be improved by optimizing the two reaction stages.

Table 3 Product compositions after each stage (wt%)

Parameter	EN14214	After Stage 1	After Stage 2 by N435	After Stage 2 by NS 88001
FAEE	>96.5	95.0	99.4	99.1
MAG	<0.8	1.25	0.10	0.17
DAG	<0.2	1.41	0.12	0.10
TAG	<0.2	0.64	0.14	0.07
FFA	<0.25	1.71	0.24	0.56

5. Concerns for large scale application

When scaling up a PBR for a larger application, pressure drop is always an important concern because it determines the feasibility, operational safety and the power input. As indicated by equation (3), the pressure drop is proportional to the length, the viscosity of the fluid and the flow velocity but varies inversely with packing material size. Therefore, the PBR is often scaled up width-wise in order to avoid the length associated pressure drop issue. On the other hand, this type of scaling way will be restricted to the extent that wide columns may vary from plug flow, with significant dispersion. Smaller packing material can give an increase in the higher reaction surface but it will greatly increase the pressure drop. Therefore, the optimal particle size should be a balance of both these issues.

As a rule the superficial velocity is always kept constant for scale up since it is such an important parameter which determines the mixing efficiency inside the PBR, particularly when components such as glycerol, the byproduct of biodiesel, can cause mass transfer limitations.

Operating window

An operating window can be a useful tool for identifying the key elements of a process and guiding the process design (Woodley and Tichener-Hooker, 1996). A conceptual operating window is given in Figure 6 in the case of transesterification in a PBR. According to the reaction characteristics the operating space (window) is defined by two variables, which are superficial velocity and cross-section area of the reactor, and three constrains, which are conversion per pass, mass transfer and pressure drop. The strategies of moving the boundaries to expand the window are summarized in Table 4. Given a certain volume of the

PBR the conversion per pass becomes unacceptable when the volumetric flowrate is above a certain level. However, this boundary can be moved upwards by improving the specific activity of the catalyst (increasing the lipase loading on the carrier or optimizing the reaction conditions). When the superficial velocity is low, the glycerol associated mass transfer problem will occur in a solvent-free system. The boundary can be moved towards the lower velocity region by using organic solvents to reduce the viscosity of the reaction mixture and improving the mutual solubility of the substrates. However, the recovery and recycle of the organic solvents will complicate the process and require extra cost. The window can be further enlarged by lowering the pressure drop limit. This can be realized by improving the mechanical stability of the carrier (increasing rigidity) or by increasing the particle size of catalyst (which will also decrease the specific activity of the catalyst leading to a lower conversion per pass).

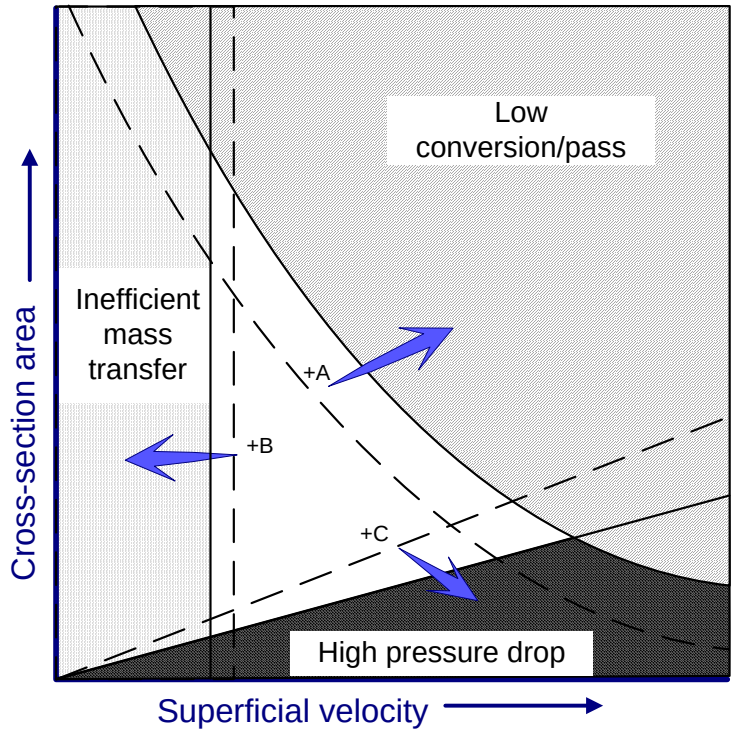

Figure 6 Operating window for PBR (A, B and C illustrate different expansions of the window, + indicates the direction as the arrow shows)

Table 4 Strategy for expanding the operating window

Strategy	Effect on expansion
Increase the lipase loading on carrier	+A
Increase temperature	+A, +B, +C
Use organic solvents	+B, +C
Change carrier for a better mechanical stability	+C
Increase the particle size	+C but -A, -B

Process options

To achieve a continuous production of biodiesel, particularly in the transesterification stage for industrial application, a process could potentially be designed based on a series of PBRs (and glycerol settling vessels between PBRs to avoid glycerol from entering the next PBR). The design should be made to make the best use of the catalyst (i.e. maximize productivity), while keeping the number of reactors to a minimum to reduce complexity and investment cost.

At the tested superficial velocities of 1.9, 3.8 and 7.6 cm min^{-1}, 14, 16 and 20 passes through a single 22 cm column (or the same number of columns in series) are required to achieve the equilibrium yield of FAEE, as indicated by the results in figure 3. This corresponds to total required bed lengths of 308, 352 and 440 cm, respectively. Increasing the column length will reduce the number of columns required, but will also increase the pressure drop. The maximum pressure drop in PBR recommended by the catalyst supplier for the sake of safety and a stable performance of catalyst is around 3 bar. The pressure drop over the 22 cm column was investigated for each of the flow velocities used and was shown to increase linearly with the flow velocity (Table 5). This indicates that it should be possible to increase the bed length for all of the investigated velocities. The maximum estimated bed length is 235, 115 and 60 cm, indicating that the minimum number of columns that can be used would be 2, 4 and 8, respectively, in order of increasing flow velocity. On the other hand, the catalyst performs considerably better at higher flow velocities, making a compromise between process complexity and catalyst cost. It is also more likely that accumulation of glycerol can cause problems in longer columns at low velocity.

Table 5 Overall productivity of first stage at different velocity

Flow velocity (cm min^{-1})	Final yield (FAEE %)	Pressure drop (bar)	Maximum bed length[a] (cm)	Total column length (cm)	Overall productivity[b]
1.9	92.4	0.28	235	286	0.016
3.8	92.4	0.58	115	352	0.026
7.6	92.8	1.1	60	440	0.042

[a.] Based on a maximum pressure drop of 3 bar; [b.] unit: gFAEE (g catalyst)$^{-1}$ min^{-1}

The results shown in figure 3 indicate that high superficial velocity is most critical at the very start of the reaction. A more detailed analysis of this can be found in Table 6, which shows the productivity of the catalyst varies over five different FAEE yield ranges for the three velocities tested. Indeed, it can be seen that the catalyst performance at 3.8 cm min^{-1} essentially matches that at 7.6 cm min^{-1} at yields higher than 20%. Based on the number of passes required in the lab and the estimated maximum bed length, it should be possible to set up a continuous PBR system based on four columns: a single 60 cm column operated at 7.6 cm min^{-1} followed by three 110 cm columns operated at 3.8 cm min^{-1}.

Since the reaction rate in the solvent-free PBR can be limited by the mass transfer to a greater extent than in an STR, especially at the start of the reaction, it could be useful to combine the two reactor types in a continuous process. One such possibility for the first stage would be to use a series of CSTRs to avoid the initial mass transfer limitation and finish with PBR(s), since mass transfer is less of an issue at high conversion and catalysts are subject to less mechanical damage in a PBR than an STR. This process will be discussed in more detail in another paper.

Table 6 Specific productivity at different conversion and velocity in the first process stage

FAEE yield (%)		Flow velocity (cm min^{-1})	Pass	Productivity (gFAEE (g catalyst)$^{-1}$ min^{-1})
Initial	**19.2**	1.9	4	0.009
Initial	**20.4**	3.8	4	0.019
Initial	**29.4**	7.6	4	0.060
19.2	**53.3**	1.9	2	0.042
20.4	**54.5**	3.8	2	0.083
29.4	**54.5**	7.6	3	0.082
58.2	**71.8**	1.9	1	0.033
54.5	**79.0**	3.8	3	0.040
54.5	**82.2**	7.6	6	0.045
71.8	**81.3**	1.9	1	0.023
65.7	**81.8**	3.8	2	0.039
71.8	**82.2**	7.6	3	0.034
81.3	**92.4**	1.9	5	0.0054
81.8	**92.4**	3.8	5	0.010
82.2	**92.8**	7.6	8	0.013

6. Conclusions

The two-stage enzymatic process proposed in this work is able to produce, in a packed bed reactor system, an ethanol-based biodiesel that is in-spec biodiesel product with respect to the main components of biodiesel (glycerides and free fatty acids), according to the EN 14214 biodiesel specifications. The efficiency of the two-stage process is comparable to other published work on methanol-based biodiesel in solvent-free systems.

The reaction rate of the first stage (transesterification) in PBR is controlled by external mass transfer to a great extent, especially in the beginning of the reaction. Additionally, the maximum conversion can be limited by accumulation of the glycerol. Both of these problems can be addressed by applying high flow velocity, and we show here that the packed bed system can in fact achieve both final yield and reaction rates that are similar to those in the best stirred tank systems.

The second stage (esterification) fulfilled the purposes of polishing the biodiesel product and improving the biodiesel yield by converting FFA and partial glycerides to FAEE. It is necessary to conduct the reaction in multiple steps with removal of water between steps to push the reaction equilibrium sufficiently far.

An evaluation of the process indicates that it should be technically and operationally feasible to scale up for the application of immobilized lipases in large-scale biodiesel production.

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PAPER 4

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Production of ethyl esters for biodiesel production using immobilized lipase in continuous stirred tank reactors

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Abstract

Immobilized lipases have been established as robust catalysts for a plethora of industrial applications, and recently, have shown potential for the production of biodiesel. However, the reaction is usually carried out in batch mode on account of simplicity. On the other hand, continuous operation offers the possibility of a stable process, which simplifies process control facilitating efficient use of manpower, making it an attractive alternative to the traditional batch process. Due to the multi-phasic (heterogeneous) nature of the transesterification system, a well-mixed system to contact the different phases and thereby aid in improving the mass transfer in the system is preferred. A continuous stirred tank reactor (CSTR) is particularly interesting as it offers the possibility of coupling the advantages of a well-mixed system with continuous operation. The main drawback of this system is that the volumetric efficiency is low. However, this effect can be mitigated by operating more than one CSTR in series. This study involves the use of immobilized lipases for continuous production of ethyl esters using multiple CSTRs in series. The sizing of the CSTRs, more specifically the flow rate and the residence times were calculated based on a Levenspiel plot, obtained from the reaction profile of a batch stirred tank reactor (BSTR) system. 2 CSTRs in series with a residence time of 71.1 min and 222 min were employed to achieve conversions of 47.1% and 74.3% respectively. A good correlation was observed between predicted and experimental data. Additionally, the efficiency of having equal-sized CSTRs in series has been benchmarked against a single batch reaction. It is proposed that the most efficient process option for the transesterification system is to use 2 CSTRs in series coupled with a continuous packed bed reactor (CPBR) to achieve equilibrium conversion.

Keywords: Biodiesel, continuous, CSTR, well-mixed, immobilized lipase

1. Introduction

The depletion of fossil fuels and increase in governmental regulations to encourage the use of blended fuels had led to an increase in the production of biofuels such as biodiesel. Biodiesel is composed of alkyl esters of fatty acids which are produced by reacting glycerides and alcohol in the presence of a catalyst (Figure 1). They have garnered considerable interest due to the multiple benefits they provide such as decrease in greenhouse gas emissions except NOx (Morris et al., 2003), increase in energy security (Hill et al., 2006), use of a renewable feedstock (Metzger, 2009) and applicability in existing transport engines (Nielsen et al., 2008; Anastopoulos et al., 2009). This in turn has led to the escalating demand for biodiesel. Consequently, the productivity of the process needs to be high, demanding efficient process technology.

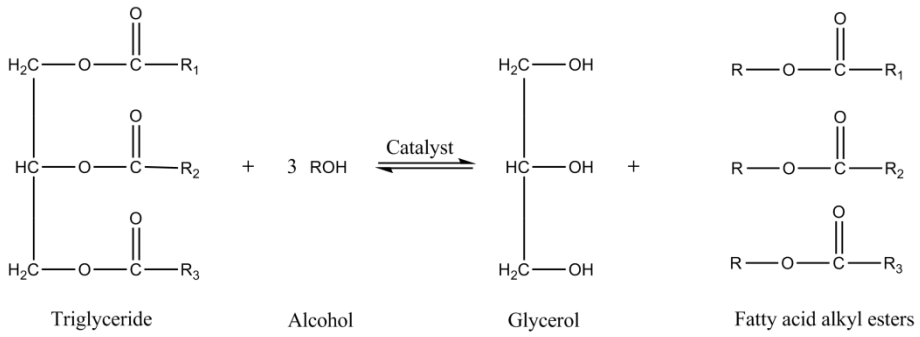

Figure 1 Transesterification reaction scheme

Biodiesel is predominantly produced in batch mode. This is not without sound reason - batch reactors are highly efficient and easy to operate, besides being capable of achieving high conversion. However, they are unsuitable for reactions with substrate inhibition (Woodley and Lilly, 1994), which is the case at higher alcohol concentrations with enzymatic biodiesel production. Additionally, at large scale, batch reactors suffer from significant downtime (Woodley, 2012). For biofuels, it is of critical importance to have high productivities; and the limitations associated with both the downtime and productivity can be mitigated to some extent by using a continuous reactor system.

Continuous production can be carried out in either plug flow (Chen et al, 2011, Xu et al., (submitted)) or well-mixed reactors (Darnoko & Cheryan, 2000). The latter in the form of a continuous stirred tank reactor (CSTR) is a well-mixed reactor, meaning that the concentration in the output stream is the same as that inside the reactor. This kind of reactor is advantageous since it is easy to operate and control. Likewise, since most reactors in the

industry are stirred tanks, it is easy to adapt to a CSTR at industrial scale. The efficient mixing of multi-phasic reaction systems in a CSTR makes them particularly suited for transesterification (the central reaction in the synthesis of biodiesel). The main disadvantage of this system is that equilibrium conversion cannot be obtained as the system operates in such a way that there is always some unreacted substrate in the effluent stream. Use of CSTRs in series improves the volumetric efficiency of the system but very large retention times would still be required to reach equilibrium conversion, necessitating the need for a combination of reactors, where a continuous packed bed reactor (CPBR) may be used subsequent to one or more CSTRs to complete the reaction. The addition of a CPBR to complete transesterification makes use of the fact that the reaction kinetics in the CPBR is the same as those in a batch reactor, thereby improving the net volumetric efficiency of the system.

This study involves the use of immobilized lipase for continuous transesterification of rapeseed oil with azeotropic ethanol (96% v/v) to fatty acid ethyl esters (FAEE) using CSTRs in series.

2. Theory

2.1 Definition of equivalence

In the production of biodiesel, a stoichiometric excess of alcohol is normally used to shift the equilibrium towards product formation (Gerpen et al., 2004). It is a common practice to apply the term equivalence (eq.) to describe the ratio of alcohol to fatty acids in the reaction system. An equivalence ratio of unity corresponds to a stoichiometric amount of alcohol and fatty acids - thus, in a reaction between oil and pure triacylglycerides (TAGs), this corresponds to a reaction mixture of 3 moles of alcohol and 1 mol of TAG.

2.2 Definition of conversion

Conversion for any chemical process is most commonly described in terms of the limiting reactant. Since the limiting reactant in transesterification of fatty acids by enzymatic catalysis is usually oil, it is appropriate to define conversion in terms of oil. Oil is comprised of TAGs, diacylglycerides (DAGs) and monoacylglycerides (MAGs), each of which is a substrate to the enzyme. Since oil should essentially contain 100% glycerides (sum of TAGs, DAGs and MAGs), conversion (X) is defined as

$$Conversion\ (X) = \frac{100 - Glyceride_t}{100} \quad (1)$$

This assumes that the mass percentage of glyceride at the start of the reaction is 100% while Glyceridet refers to the mass percentage of glyceride at time t.

2.3 Transfer from BSTR to CSTR

A BSTR is well-mixed and operates under non-steady state conditions, where the conversion changes with time until equilibrium is reached - in other words, there is consumption of reactants and accumulation of products in the reactor. This implies that the conversion is a function of time. In contrast, continuous reactors operate at steady-state (not including the start-up phase) and the conversion is independent of time. Hence, the conversion in a CSTR is a function of the residence time within the reactor, which is fixed with respect to the ratio of working volume to that of the volumetric flow rate.

Batch reaction data can be used to establish the correlation between reaction rate and conversion for a given set of reaction conditions. This can be done graphically by drawing the tangent to the plot of conversion data against reaction time at different levels of conversion, or by fitting one or several mathematical function(s) to the experimental data and then deriving an empirical rate expression. This information can then be combined with the reactor mass balance to predict the performance of a CSTR.

The design equation of a CSTR is derived from the mass balance of the system taking the mass of the working reactor volume into account. The equation is derived based on the limiting reactant (glycerides), which has been used for this study (Levenspiel, 1998). The mass balance of glycerides can be given by the equation:

$$Input = Output + Consumed\ in\ reaction + Accumulation \quad (2)$$

At steady state, accumulation term is zero.

Input of glycerides to the system can be given as

$$Input_{Glycerides}(mass/time) = F_{Glycerides0} \cdot (1 - X_{Glycerides0}) \quad (3)$$

where F is the flow rate in g/unit time, XGlycerides0 is the conversion at t=0.

Conversion at t=0, for the first reactor is 0. Hence equation 3 becomes

$$Input_{Glycerides}(mass/time)=F_{Glycerides0} \quad (4)$$

Similarly, a mass balance for the output of the glycerides could be represented by the equation

$$Output_{Glycerides}(mass/time) = F_{Glycerides} = F_{Glycerides\,0}(1 - X_{Glycerides}) \quad (5)$$

The rate of disappearance can be represented as

$$Disappearance of\ glycerides = (-r_{Glycerides})V \quad (6)$$

where V denotes the volume of the reactor and r the rate of utilization of glycerides. The units of rate would be mass% min-1.

Substituting equations 4, 5 and 6 in 2,

$$F_{Glycerides0}X_{Glycerides} = (-r_{Glycerides})V \quad (7)$$

Rearranging,

$$\frac{V}{F_{Glycerides_0}} = X_{Glycerides} \cdot \frac{1}{-r_{Glycerides}} \quad (8)$$

Equation 7 is known as the design equation for a CSTR, which shows that the required reactor volume for a given flow rate is proportional to the desired conversion level and inversely proportional to the reaction rate. A plot of 1/r as a function of conversion (X) is commonly known as the Levenspiel plot (Figure 2). At a fixed conversion point, the area enclosed by the rectangle (determined by the 1/r at the operating point and the change in conversion) gives the ratio of mass of glycerides to the flow rate of glycerides (mass of glycerides/unit time) into the system. This is equal to the residence time required. Therefore, for a fixed volume, the flow rate of glycerides can be calculated. The plot can also be used to size more than one CSTR in series. Furthermore, this plot is useful for designing plug-flow reactors, where the area under the curve gives the residence time in a tubular reactor.

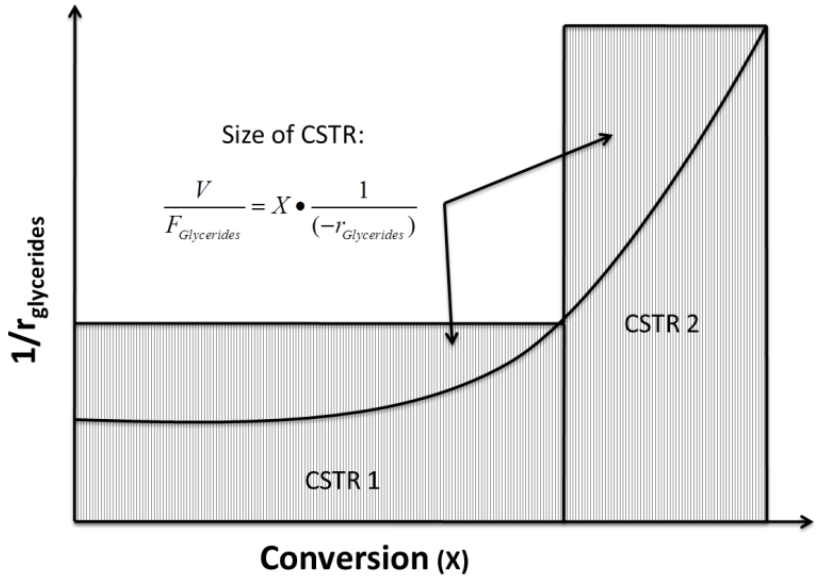

Figure 2 Levenspiel plot for determining the residence time in the reactor. The shaded area represents the residence time for a given flowrate through a CSTR

3. Materials and Methods

3.1 Sample preparation and HPLC

Samples were diluted for subsequent analysis by HPLC. 55 µl was pipetted from the oil phase and added to 500 µl of sample solvent, n-heptane (Sigma Aldrich A/S, Steinheim, Germany)). The samples were then centrifuged at 14,500 rpm for 10 min to separate catalyst and glycerol and prevent them from entering the column. The supernatant was diluted 100-fold, thus preparing the samples for HPLC analysis and 40 µl of the prepared sample was injected in the HPLC for analysis. Multiple calibrations indicate an error of ±5%.

The samples were analyzed by HPLC (Ultimate 3000, Dionex A/S, Hvidovre, Denmark) for FAEE and glyceride concentration. The HPLC was equipped with a cyanopropyl column (Discovery® Cyano, Sigma Aldrich A/S, Brøndby, Denmark) (0.25 x 0.004 m), U3000 autosampler, TCC-3000SD column oven, U3400A quaternary pump modules and a Corona® Charged Aerosol Detector (Thermo Scientific Dionex, Chelmsford, MA, USA). Nitrogen at a flow pressure of 2.41 kPa (35 psi) was for detection. A binary gradient program was applied using Phase A: 99.6% n-heptane, 0.4% acetic acid and phase B: 99.6% methyl-tert-butyl ether and 0.4% acetic acid (Foglia, 1997).

3.2 Materials

Rapeseed oil was kindly donated by Emmelev A/S (Odense, Denmark). Transesterification was performed using azeoptropic ethanol (96% v/v), purchased from Kemetyl A/S, Køge, Denmark.

3.2.1 Biocatalyst

All reactions were carried out with pre-sieved (500 µm) *Thermomyces lanuginosus* lipase immobilized on a polymeric resin (TLL, an experimental catalyst) (NS 40077) kindly donated by Novozymes A/S (Bagsværd, Denmark).

3.3 Reactors

All reactions in well-mixed reactors were carried out in a 1L glass reactor where the feed pipes functioned as baffles (ACE Glass, Vineland, NJ, USA), which was thermostated at 35°C. The agitator is a glass shaft with a 4-blade flat blade impeller (0.011*0.032 m) made of PTFE. A motor (IKA® RW 20 Digital, Staufen, Germany) was connected to the shaft via a coupling that enabled mixing in the reactor. The stirring speed was set to 200 rpm. 5% (m/m) enzyme loading (which corresponds approximately to 13-15% (v dry catalyst/v substrate) depending on the composition of the substrate) was used in all these reactions.

3.3.1 CSTR

CSTR experiments were carried out in the same reactor used to collect batch kinetic data. Diaphragm membrane pumps (STEPDOS 03, KNF, Freiburg, Germany) were used to feed pre-heated oil (35° C) and ethanol while a peristaltic pump (Watson Marlow A/S, Ringsted, Denmark) was used to remove effluent. The CSTR was equipped with a filter made of a metal mesh of 100 microns, placed at the bottom of the reactor. This helped to retain the catalyst beads inside the reactor.

3.4 Batch Experiments

500g of rapeseed oil was processed with 1.0 and 1.5 equivalents of azeotropic ethanol corresponding to a volume of 93.8 ml and 139 ml respectively. 25g of pre-soaked (soaked overnight in oil) catalyst was used to catalyze the reaction. The reactions were carried out for 1440 min and the samples were taken regularly at the start of the reaction (0m, 5m, 10min,

15min, 20min and 30min), and subsequently every 15mins up to 4 hours and finally every 30 min up to 8h. 24h and 25h samples were taken to ensure that equilibrium was attained.

3.5 CSTR sizing

Batch reaction data were used to make a Levenspiel plot. A plot of glyceride conversion vs. time was made for both 1.0 and 1.5 eq. reactions and these were used to determine the reaction rate at defined conversion points. The plots were then divided into 4 sections in such a way that the last point of one section was the start of the next. Each section was fitted to a second order polynomial. The derivative of the equation at a particular conversion point gave the reaction rate at that point. The Levenspiel plot was then plotted with these reaction rates and the corresponding conversions. Two conversion points were chosen for the operation of the 1^{st} and 2^{nd} CSTR stages, more specifically, 47.1% and 74.3%.

3.5.1 Estimation of performance of CSTRs in series

In order to compare the performance of the CSTR system with that of a BSTR, the Levenspiel plot was used to determine the residence times required for the 1 CSTR, 2 or 3 equal sized CSTRs in series based on the operating conversion levels. Optimum conversion points were determined such that the residence time in the first reactor was the same as the others in series. Linear interpolation between the calculated points of conversion was used in order to determine the reaction rates at these operating points. While the 1.0 Eq. plot was used for conversions up to 75%, 1.5 Eq. plot was used for higher conversions (up to 90%).

3.6 Control of ethanol concentration

A batch reaction was run to 70% conversion, the catalyst removed and the glycerol phase was separated from the oil phase. 5g of the oil phase and 1g of the glycerol phase were subjected to evaporation at 100°C overnight to establish the distribution of volatiles between the two phases. The loss in weight was measured as the loss in ethanol from each phase. To compensate for the ethanol loss in glycerol separation between CSTR stages 2 and 3, the effluent from CSTR 2 was first analysed by HPLC. The total amount of remaining ethanol was calculated based on the product composition, and the amount of ethanol in the oil phase was estimated using the previously established distribution. Ethanol was then added to the reaction mixture to match the concentration in a batch experiment with 1.5 eq ethanol at that conversion. In practice, this means that for an effluent with 70% conversion, the total ethanol

amount was adjusted to 2.6 Eq. with respect to the glycerides remaining at the end of the reaction.

3.7 Evaluation of non-steady state behaviour

The time taken to reach steady state largely depends on the kinetics of the reaction; specifically, the order of the reaction and the rate constant(s). For reactions that follow, or approximately adhere to, first order reaction kinetics, the rate constant can be determined by plotting the logarithm of the reactant concentration against t. The slope of the line gives the rate constant. The time taken to reach 99% of the steady state can then be calculated using the equation,

$$t_s = 4.6\frac{\tau}{1+k\tau} \qquad (9)$$

where, t_s is the time taken to reach steady state, τ is the residence time and k is the rate constant (Fogler, 2006).

4. Results

4.1 BSTR

The retention time in the CSTR was calculated based on reaction rate data obtained from BSTR reactions. Minimum ethanol feeding was restricted to 1.0 Eq. because lower concentrations of feed would not yield viable equilibrium conditions, leading ultimately to a very inefficient CSTR system. The reaction profile of BSTR systems operating at 1.0 and 1.5 Eq. systems are shown in Figure 3 and the corresponding Levenspiel plot are depicted in Figure 4. A further increase of ethanol to 2.0 Eq. does not provide a further increase in reaction rate, possibly due to inhibition by ethanol (data not shown). Therefore, ethanol concentrations above 1.5 Eq. have not been considered. Additionally, higher ethanol concentrations would lead to lower catalyst life time.

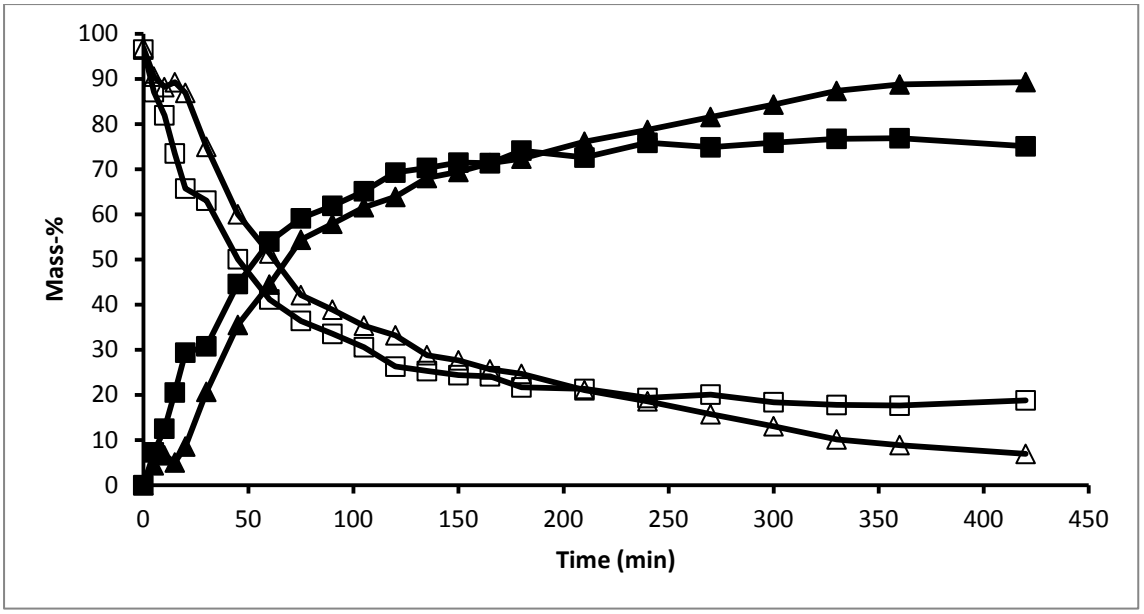

Figure 3 Consumption of glycerides and formation of FAEE in a BSTR as a function of time. (■)- 1.0 Eq. FAEE, (□) – 1.0 Eq. Glycerides, (▲)-1.5 Eq. FAEE, (Δ) – 1.5 Eq. Glycerides

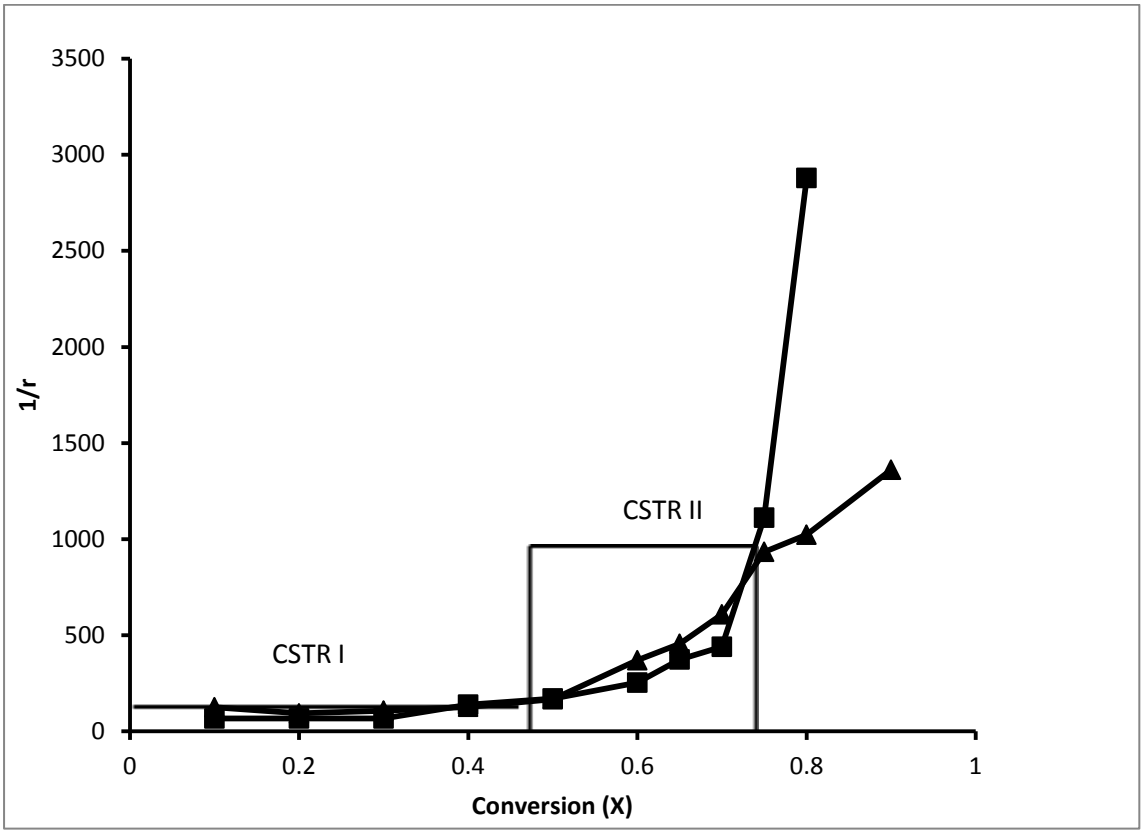

Figure 4 This graph represents the inverse of reaction rates with respect to conversion for varying initial ethanol equivalence (Levenspiel plot). (▲)-1.5 Eq. EtOH, (■) – 1.0 Eq. EtOH

From Figure 4 it can be seen that the reaction rate is similar for both 1.0 and 1.5 Eq. systems up to values of about 70% conversion which renders the use of the higher concentration of ethanol in the system unnecessary at operating conversion beneath this level. Also, with addition of 1.5 Eq. ethanol, a lag phase (seen in Figure 5) is predominant, which could affect the start-up of the reaction system. Interestingly the 1.5 eq. system gives higher equilibrium conversion, and there is a significant difference between the reaction rates of 1.0 and 1.5 eq. reactions at higher conversions as expected, indicating that additional ethanol should be supplied to reactors operating at such conditions. The areas enclosed by the black rectangles in the Figure 5 depict the residence times chosen to operate and validate the CSTR.

4.2 Validation of CSTR

The retention times in the CSTR were based on the Levenspiel plot as described by Fogler (Fogler, 2006). From the Levenspiel plot, it can be seen that the 1/r value increases marginally up to 50% conversion and then increases strongly. It was therefore chosen to have 2 CSTRs in series with the first one run at 47.1% conversion (Figure 5) while the second one was run at 74.3% conversion (Figure 6), which is the upper limit for using the 1 eq. reaction system. The corresponding retention times in the 2 reactors were calculated to be 71.1 min and 222 min respectively. In an ideal, well-mixed system, there should be good correlation between the predictions and experimental values (Carleysmith and Lilly, 1979) and this was confirmed - the first stage of CSTR (Figure 5), gave a 48.3% conversion which is close to the expected 47.1%.

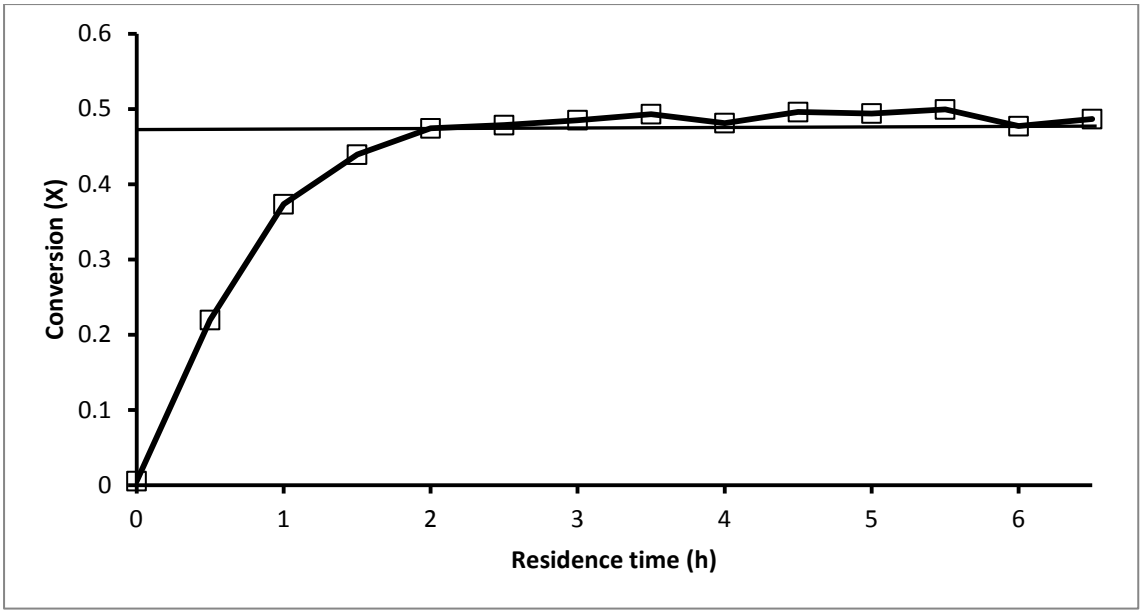

Figure 5 CSTR Stage I – 47.1% Conversion. (□)-Glyceride conversion. The horizontal black line indicates the calculated conversion at steady state.

As can be seen from the Figure 6, the conversion in the CSTR can be divided into 2 stages, a dynamic stage in which the conversion increases and a steady state region where there is constant conversion. The duration of the start-up region largely depends on the kinetics of the reaction inside the reactor. At the conversion point of the first reactor, the reaction follows first order kinetics and hence the time taken to reach steady state can be calculated using Equation 8. The rate constant for this operating region was calculated to be 0.0129 min^{-1}. The time needed to reach 99% of the steady state value was calculated to be 2.8 h, which appears to correlate well with the experimental value (approx. 3 h). This supports the assumption that the system was well mixed and there was no spatial variation of concentration within the reactor.

For the 2nd CSTR stage (Figure 6), the average conversion achieved at steady-state was 74.3%, which is close to the estimated conversion for this residence time. The feed consists of a mixture that has a 3.5% increase in conversion compared to the effluent from the first reactor. This 3.5% increase in conversion is assumed to be due to the presence of residual enzyme activity in the stored mixture (potentially due to leaching). This effect would be overcome by running the CSTRs continuously without a time-lag between the stages.

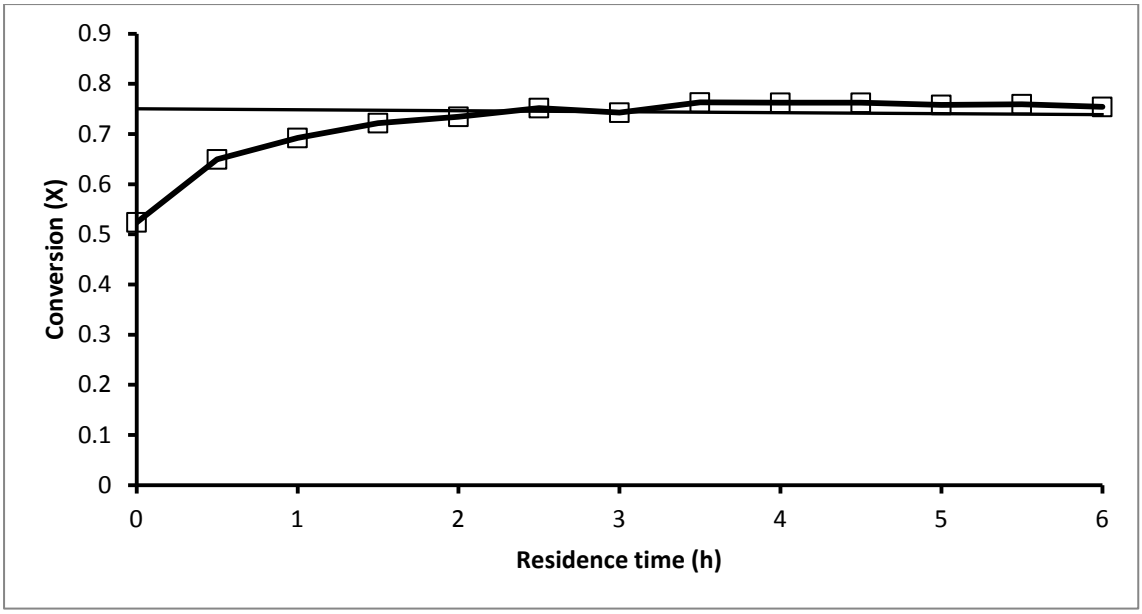

Figure 6 CSTR Stage II – 74.3% conversion. (□)-Glyceride conversion. The horizontal black line indicates the predicted conversion.

4.3 Glycerol separation and control of ethanol concentration

After achieving 70% conversion, a glycerol separation stage was incorporated. This was done for 2 reasons. First, in order to push the equilibrium towards product formation. Secondly, glycerol is known to lower catalyst activity by clogging the catalyst (Lam et al., 2010; Xu et al., 2011). However, since ethanol is soluble in glycerol, a significant amount of ethanol is lost by the separation (approximately 70% was lost in the glycerol phase) and was compensated as explained in the methods section.

4.4 CSTR 3

A third CSTR stage (Figure 7) was operated at the lowest flowrate possible in the current set-up, in order to estimate the maximum conversion achievable in this dilution-rate limited system. It was hypothesised that this stage would cause considerable reduction in glycerides that would end up going into the following polishing stages. As can be seen, only a small increase in conversion (approx. an additional 10%) is achieved by using this extra CSTR stage. The usefulness of this reduction is dependent on the requirements of subsequent processing stages.

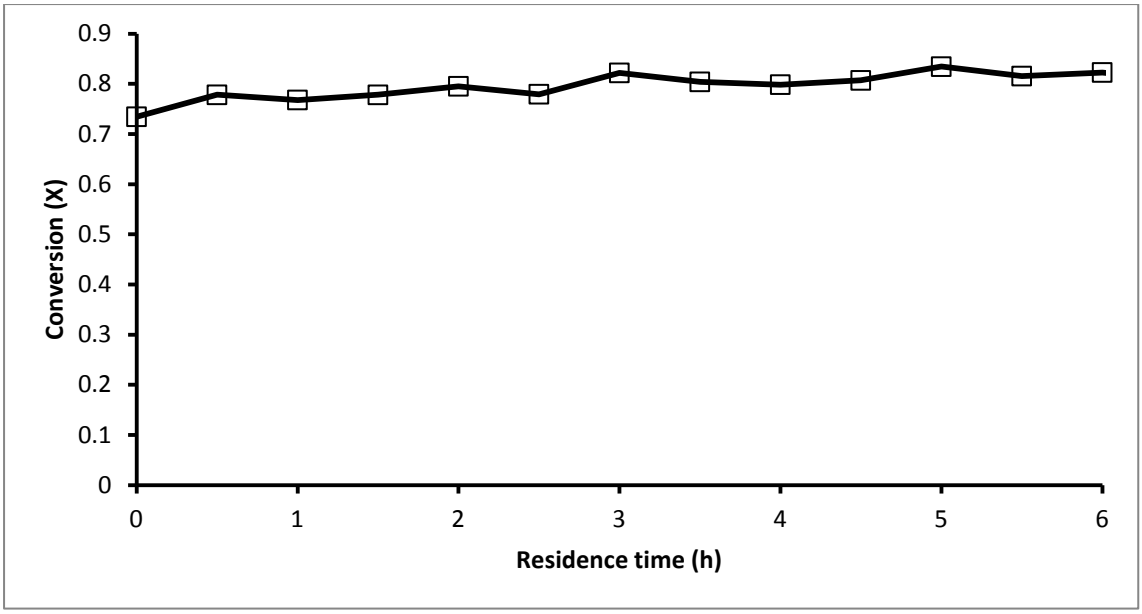

Figure 7 CSTR stage III (□)-Glyceride conversion

5. Discussions

This study has shown that a CSTR can efficiently handle a multi-phasic reaction system such as transesterification catalysed by an immobilized lipase. Further, it has been shown that established methods for designing CSTRs based on batch reaction data are applicable to this system, which has been validated by operating 2 CSTRs in series at defined experimental set points. To further illustrate the potential efficiency of a continuous system consisting of CSTRs in series, compared to the efficiency of a batch reaction system, an efficiency curve (Figure 8) was generated. This was obtained by comparing the residence times required to reach a particular conversion in a CSTR system to the reaction time required to reach the same point in a batch system. When the efficiencies in the batch and the CSTR systems are the same, the ratio of the residence times in the reactors will be equal to 1. The total required residence times required to reach different levels of conversion was calculated for the three reaction systems by using the Levenspiel plot for 1.0 Eq. up to 75% conversion and the 1.5 Eq. plot for higher conversions. These were then compared to the reaction times required to reach the same levels of conversion in batch. For conversion levels higher than 75 %, batch reaction times were estimated by combining 1 and 1.5 eq batch data to simulate a two-step fed-batch system, with 0.5 additional equivalents of ethanol added at 75 % conversion/174 min. The small lag phase observed in the 1.5 Eq. reaction was compensated for when calculating the efficiency.

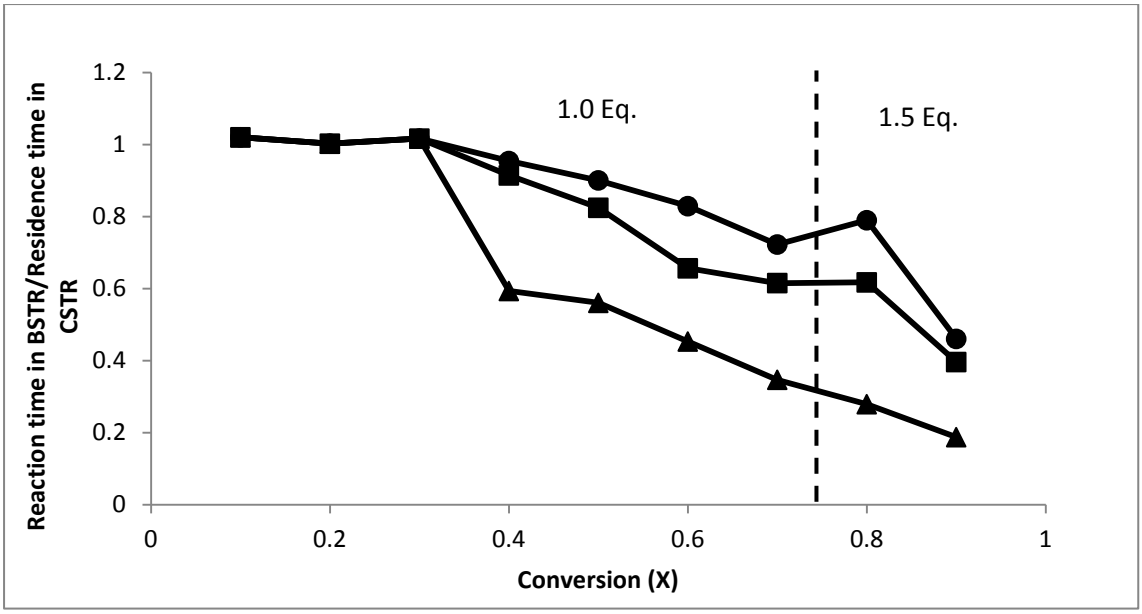

Figure 8 Efficiency of CSTR system with respect to batch reactor. (▲)-1 CSTR, (■) - 2CSTRs in series, (●)-3 CSTRs in series. The dashed line indicates the shift from 1.0 Eq. to 1.5 Eq.

From the plot, it can be seen that the efficiency of a CSTR system is the same as that of the batch system at lower conversions. However, as the conversion increases, the batch system is increasingly efficient. It is also apparent that operating a single CSTR to obtain high conversion is inefficient. A slight increase in efficiency can be seen at a conversion of 80%. This is an effect of shifting from 1.0 Eq. to 1.5 Eq. systems at that conversion point. Additionally, the improvement of volumetric efficiency of having CSTRs in series can be seen. While the volumetric efficiency is improved (the degree of improvement is dependent on the conversion), addition of a third CSTR would add to the complexity and the cost of the process.

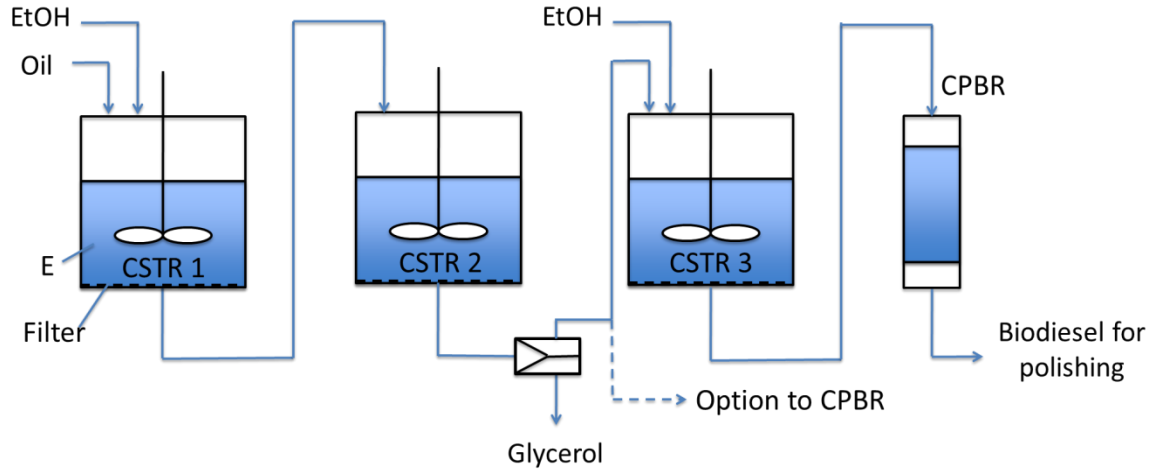

Figure 9 Process options for continuous biodiesel production

In order to facilitate further processing of biodiesel (to bring it in specification (Knothe, 2006)), high conversions from the transesterification stages are desired. However, to achieve very high conversions in a CSTR system it would have to be operated close to equilibrium, which is not practical. The ideal solution to attain high conversion is to shift from a CSTR system to a CPBR system that operates with an efficiency similar to that of a batch reactor. Xu et. al. have shown that it is possible to operate a CPBR system so that it mimics the efficiency of a BSTR in the transesterification reaction (Xu et al., (submitted)). An example of a system with CSTRs in series with CPBR is depicted in Figure 9. Assuming that the CPBR can be operated at similar efficiency as that of a BSTR, three process alternatives have been considered (i) 3 CSTRs in series, (ii) 2 CSTRs in series with a CPBR and (iii) 3 CSTRs in series with a CPBR and their corresponding processing intensities have been plotted (Figure 10).

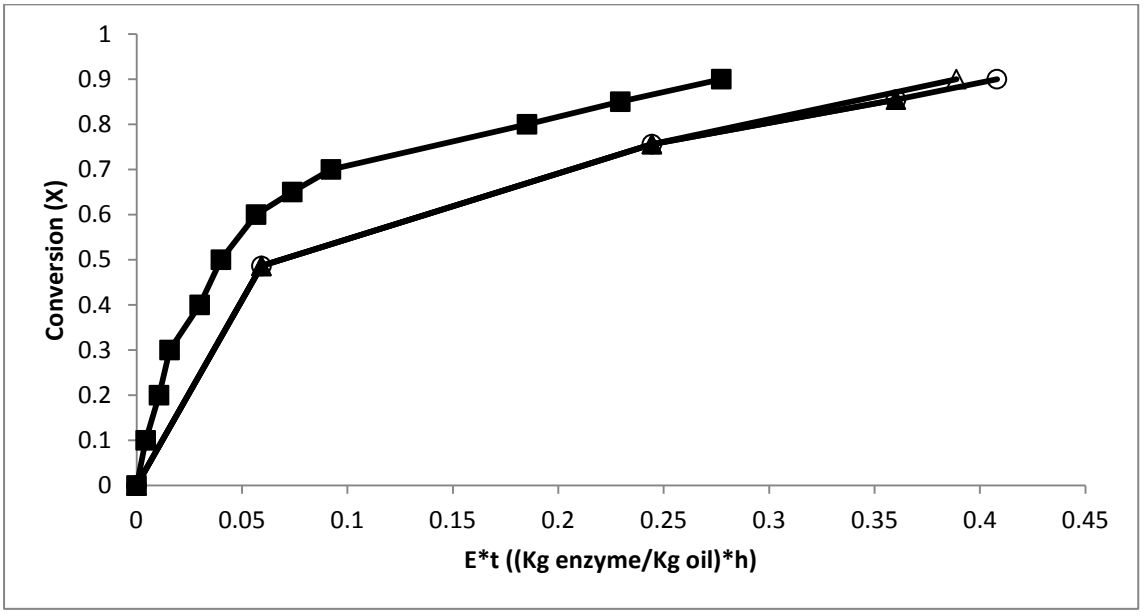

Figure 10 Comparision of process efficiency in the continuous process alternatives. (■)-batch, (▲)-3 CSTRs in series, (Δ)-2 CSTR+CPBR, (○)-3 CSTR+CPBR

Processing intensity can be defined as the concentration of enzyme used and the time taken for the system to reach a particular conversion point. Therefore, a lower required processing intensity indicates a better-performing of the system. For the process options, the amount of enzyme used and the time taken for a particular conversion (in case of the batch reaction) and the residence times (for CSTR) were used from the experimental data to obtain the plot in Figure 10. The E*t value for the CPBRs were obtained by assuming that they perform with a similar efficiency as that of a BSTR.

As can be seen from Figure 10, the batch system has the least processing intensity and hence the best performance. However, amongst the continuous process options, the use of 2CSTRs in series with a CPBR has the best performance for reaching a conversion of 90%. Although it is possible to replace the continuous systems with a CPBR system which operates at similar processing intensities as that of a BSTR, high linear velocities were required to reach approximately 70% conversion. However, the use of a CPBR has proved to be difficult due to a coupled effect of mass transfer limitations (at initial conditions) and glycerol accumulation in the column (at higher conversions) (Xu et al., (submitted)). Whilst the latter problem can be suppressed by use of high flow rates, the former limitation is more difficult to address in a CPBR. Therefore, replacing this conversion region with a CSTR and using a CPBR to complete transesterification, could provide an efficient continuous process option for the production of biodiesel.

6. Conclusions

Use of CSTRs in series for continuous production of ethyl esters catalyzed by immobilized lipase has been established in this study. The advantages of a continuous operation over a batch mode make it a viable alternative for production of biodiesel.

Secondly, CSTR sizing using the Levenspiel plot has been executed and the methodology validated in this study. Furthermore, the operation of more than one CSTR in series has been established and the experimental data has shown good correlation with the theoretical predictions.

A good correlation between the predictions and actual performance was observed indicating the presence of a well-mixed system where there is no spatial variation of concentration. This further shows that the CSTR is efficient in handling a multi-phasic reaction system as in transesterification. Another important implication of a well-mixed system is that there is no short-circuiting of the reactants directly to the product stream.

Use of CSTRs in series improves the net volumetric efficiency of the system. However, it was seen that very large retention times were required when the system reached conversions close to equilibrium. Hence, to achieve high conversions in the transesterification stage, it has been suggested that CSTRs in series be employed in conjunction with CPBR for completing the transesterification reaction. This would further improve the volumetric efficiency of the

system. Nonetheless, a polishing stage might be necessary to bring the biodiesel that has been produced in-spec.

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